Refining Genotype-Phenotype Correlation In Epidermolysis Bullosa

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Refining Genotype-Phenotype Correlation In Epidermolysis Bullosa

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ABSTRACT

Dystrophic epidermolysis bullosa results from mutations in *COL7A1* that encodes collagen VII, the major component of anchoring fibrils. General paradigms have emerged attributing dominant DEB to heterozygous glycine substitutions and recessive DEB to nonsense, frameshift or splice site mutations on both *COL7A1* alleles. Several aspects of the genotype-phenotype heterogeneity encountered in DEB remain unexplained, although genetic, epigenetic and environmental modulators have been implicated. In this thesis, various aspects of DEB were studied, in a bid to refine genotype-phenotype correlation. A detailed analysis of missense GS and non-GS mutations identified 57 novel mutations and was in-keeping with the general established paradigms. Unique clinical entities such as EB-pr and BDN were studied. The role of the matrix metalloproteinase-1 promoter polymorphism, -1607 1G/2G, on disease modification in EB-pr was explored, but was shown to be an unlikely modulator. A large study of BDN, highlighted that intracytoplasmic retention of collagen VII and stellate bodies were not exclusive to BDN and can be associated with various subtypes of DEB, non-EB cases and normal skin. The first case of revertant mosaicism in DEB was studied revealing intragenic cross-over as the underlying mechanism. Finally, intradermal injection of allogeneic fibroblasts was shown to result in increased and sustained expression of *COL7A1* possibly through the upregulation of HB-EGF. Recognition of disease modifiers in DEB and refinement of genotype–phenotype correlation will not only further our understanding of DEB but will have implications on diagnosis, counselling and prognosis through patient specific and targeted therapy.

To John and Jemma

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ABBREVIATIONS

AA	amino acid
AD	autosomal dominant
AF	anchoring fibril
AP	activator protein
AR	autosomal recessive
BDN	bullous dermolysis of the newborn
BM	basement membrane
BM	bone marrow
BMZ	basement membrane zone
BPAG	bullous pemphigoid antigen
cDNA	complementary deoxyribonucleic acid
CMP	cartilage matrix proteins
CRISPR	clustered regulatory interspaced short palindromic repeats
DEB	dystrophic epidermolysis bullosa
DDEB	dominant dystrophic epidermolysis bullosa
DDEB-na	dominant dystrophic epidermolysis bullosa- nails only
DDEB-pt	pretibial dominant dystrophic epidermolysis bullosa
DEJ	dermoepidermal junction
DIF	direct immunofluorescence
DMEM	Dulbecco’s modified Eagle’s medium
DNA	deoxyribonucleic acid
EB	epidermolysis bullosa

EB-Pr	epidermolysis bullosa pruriginosa
EBS	epidermolysis bullosa simplex
EBS-MD	epidermolysis bullosa simplex with muscular dystrophy
ECM	extra-cellular matrix
EDTA	ethylenediaminetetracetic acid
EM	electron microscopy
ENCODE	encyclopaedia of DNA elements
ESE	exonic splicing enhancer
ETS	E26 transformation specific
FBS	foetal bovine serum
FCS	foetal calf serum
GABEB	generalized atrophic benign epidermolysis bullosa
gDNA	genomic deoxyribonucleic acid
GS	glycine substitution
HaCaT cells	human adult low calcium high temperature cells
HB-EGF	human heparin binding epidermal growth factor
HD	hemidesmosomes
HiDi formadide	highly deiodinised formadide
HJEB	Herlitz junctional epidermolysis bullosa
HLA	human leukocyte antigen
HMGB1	high mobility group box 1
IF	immunofluorescence
IgE	immunoglobulin E
IL	interleukin
iPSCs	induced pluripotent stem cells

IVS	intervening sequence (intron)
JEB	junctional epidermolysis bullosa
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MS	missense
MSC	mesenchymal stromal cells
NaBu	sodium butyrate
NC	non-collagenous
NHJEB	non- Herlitz junctional epidermolysis bullosa
NHS	normal human skin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PTC	premature termination codon
qRT-PCR	quantitative real-time polymerase chain reaction
RDEB	recessive dystrophic epidermolysis bullosa
RDEB-I	recessive dystrophic epidermolysis bullosa-inversa
RDEB-O	recessive dystrophic epidermolysis bullosa-other
RDEB-pt	pretibial recessive dystrophic epidermolysis bullosa
RDEB-sev gen	severe generalised recessive dystrophic epidermolysis bullosa
RER	rough endoplasmic reticulum
RM	revertant mosaicism
RNA	ribonucleic acid
RT	reverse transcription
SCC	squamous cell carcinomas

SCID	severe combined immunodeficiency
SE	standard error
SMaRT	spliceosome-mediated RNA trans-splicing
SR	serine-rich
SS	splice site
TALEN	transcription activator-like effector nucleases
TBDN	transient bullous dermolysis of the newborn
TBE	tris-borate- ethylenediaminetetracetic acid
TBP	TaTa box binding protein
TEM	transmission electron microscopy
TGF	transforming growth factor
THC	triple helix collagenous domain
TIMP	tissue inhibitors of metalloproteinases
TNF	tumour necrosis factor
UNG	uracil-N-glycosylase
USSC	unrestricted somatic stem cells
UV	ultraviolet

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DECLARATION

This thesis contains no material that has been accepted for the award of other degree or diploma in any university. The work contained in this thesis is the work of the author unless stated otherwise and as such the copyright of this thesis rests with the author and no quotations from the information derived from it may be published without the prior consent of the author.

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Chapter 1

General Introduction

Epidermolysis Bullosa (EB) is a rare genetic skin disorder characterised by skin fragility and blistering of the skin and mucous membranes following minimal trauma (Fine *et al.*, 2014). It affects close to half a million patients worldwide (Uitto, 2009) and has been attributed to mutations in at least 18 genes encoding various structural proteins crucial for skin integrity. Recognition of the associated immunohistochemical and ultrastructural alterations to normal skin structure, particularly to the basement membrane zone (BMZ), has formed the basis of our understanding of EB pathology.

1.1 Normal skin structure

The skin is the largest organ in the body and is estimated to cover a surface area of about $2m^2$ (McGrath and Uitto, 2010). Not only does it act as a biological barrier to external pathogens and stresses but it also plays an integral role in homeostasis, thermoregulation and defence against ultraviolet-induced DNA damage. In addition, it has important immunological, endocrinological and sensory functions (McGrath and Uitto, 2010).

The skin structure, which is 1.5-4mm thick, is composed of 2 layers; the epidermis and dermis, that overly the subcutaneous tissue. The ectodermally derived keratinocytes constitute the majority of the epidermis which is arranged in 4 distinct layers following the direction of keratinocyte differentiation; the basal, spinous, granular and corneal layers. In order to maintain the unique epidermal structure and alignment, the keratinocytes possess a cytoskeleton composed of keratin intermediate filaments. These are important for maintaining mechanical strength and linkage to desmosomal

junctions involved in cell adhesion and signalling (Kirfel *et al.*, 2003; Lee *et al.*, 2006; Menon *et al.*, 1985; Menon *et al.*, 1992; Elias, 2012; Elias, 1983; Jackson *et al.*, 1993; Braff *et al.*, 2005; Oren *et al.*, 2003; Sorrell and Caplan, 2004).

The dermis on the other hand, is composed of cells, appendageal structures, nerves and vessels embedded in an extra-cellular matrix (ECM). The latter is formed mainly of collagen and elastic connective tissue fibres that confer tensile and elastic properties, respectively. The dermis also contains non-collagenous proteins of importance to cell adhesion and mobility, as well as non-fibrous ground substance that include proteoglycans and glycosaminoglycans.

1.2 The cutaneous basement membrane zone

In between the epidermis and dermis lies the BMZ, a complex structure of interlacing macromolecules integral for fortifying dermal-epidermal adhesion and maintaining skin integrity. This biological scaffold extends from the basal keratinocyte layer to the papillary dermis and includes the anchoring filaments, hemidesmosomes and anchoring fibrils (Figure 1.1). It is formed of an upper lamina lucida, which directly abuts the plasma membranes of the basal keratinocytes and a lower lamina densa that interacts with the mesenchymal matrix of the upper dermis (Fuchs, 2007) (Figure 1.2). Disruption in any of the macromolecular links of the BMZ (Figure 1.3) leads to various degrees of skin fragility with resultant distinctive phenotypes (Uitto and Christiano, 1992)

Figure 1.1 Diagrammatic representation of the basement membrane zone. The components of the BMZ are shown including the tonofilaments, hemidesmosomes (HD), lamina lucida, lamina densa and anchoring fibrils.

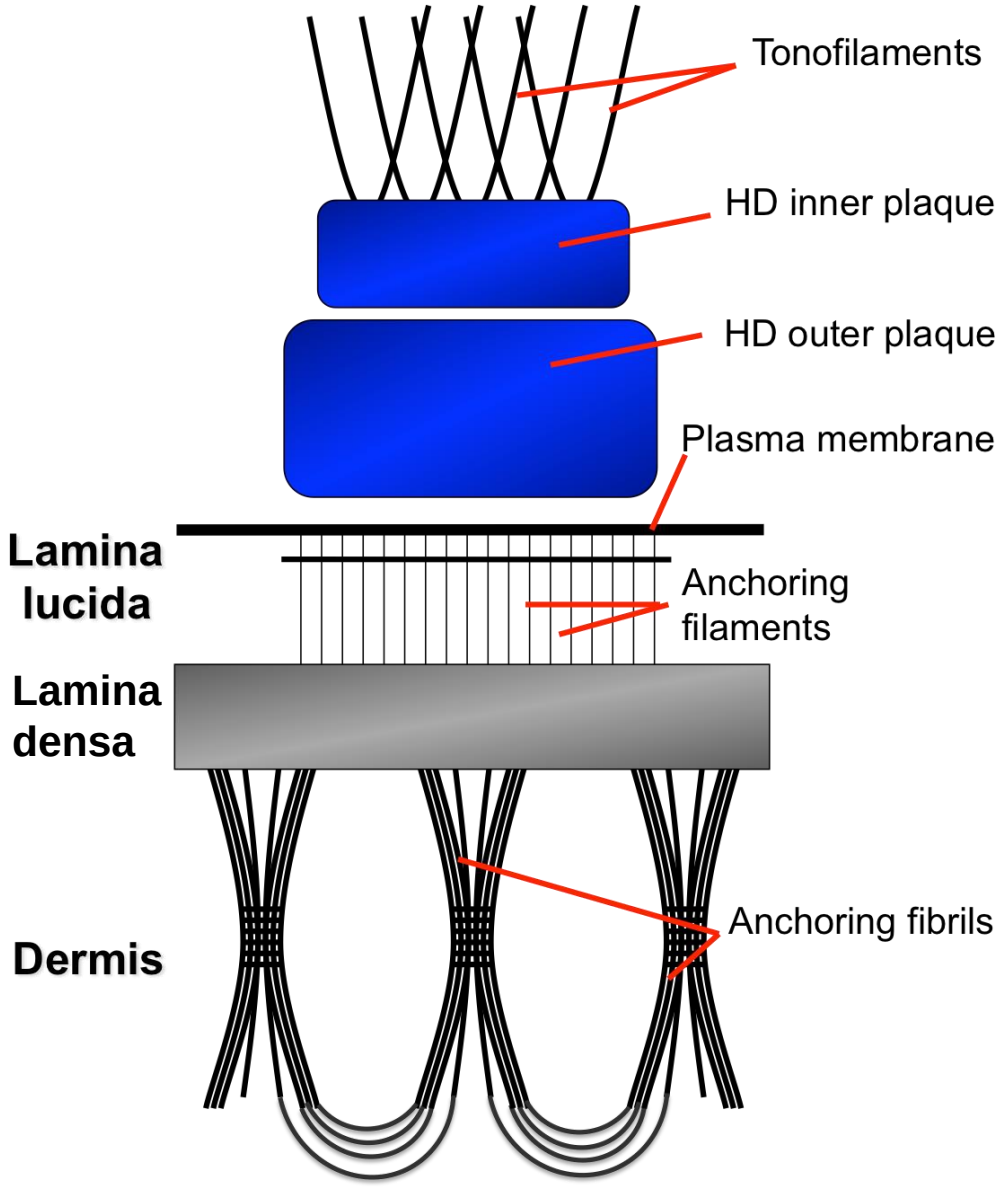


Figure 1.2 Anchoring fibril morphology at the basement membrane zone. (A) Schematic representation of the hoop-like anchoring fibrils. (B) Transmission electron microscopy (TEM) shows the lamina lucida and lamina densa, as well as the anchoring fibrils (arrows) that extend from the BMZ into the papillary dermis (bar= 0.2μm).

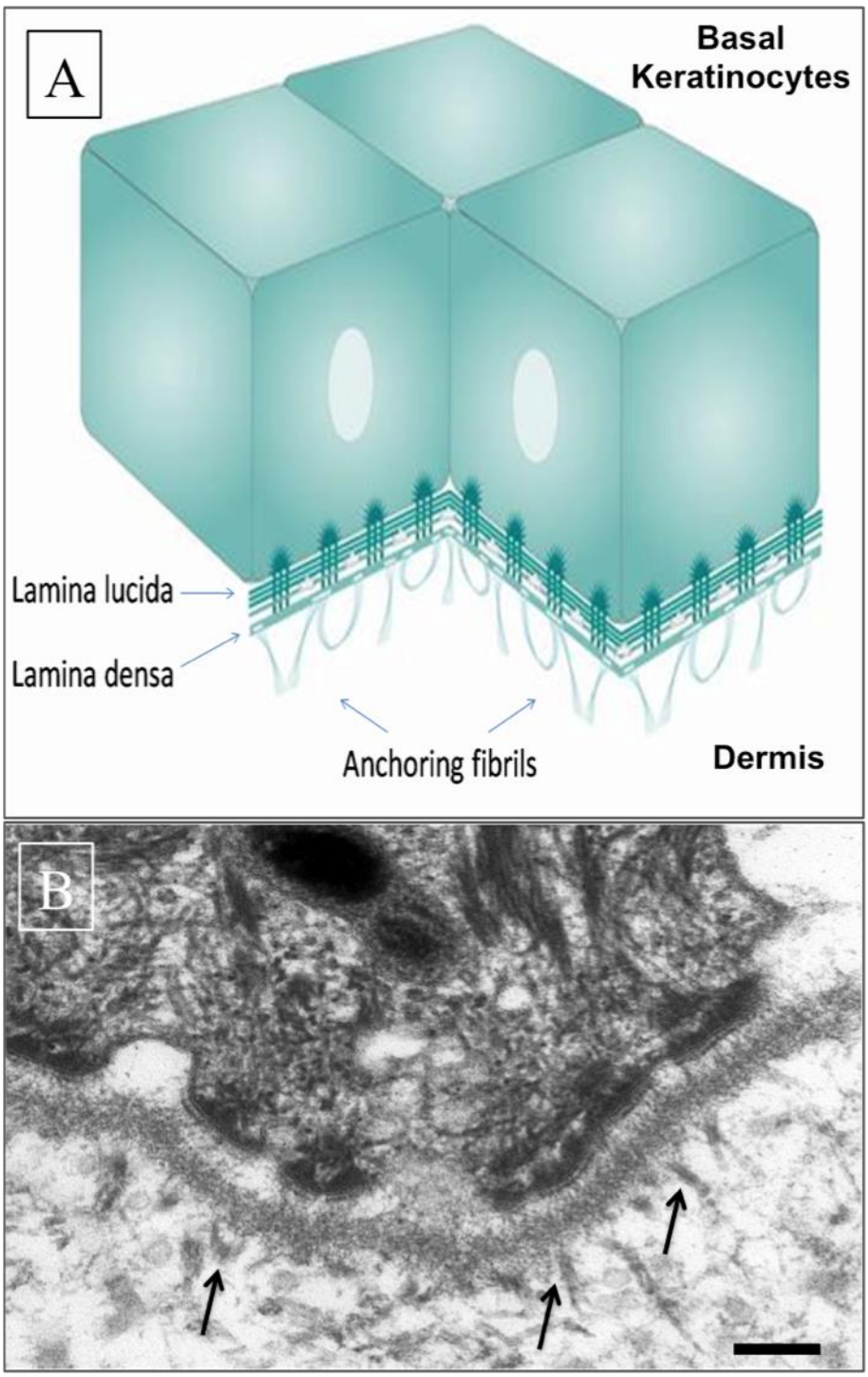
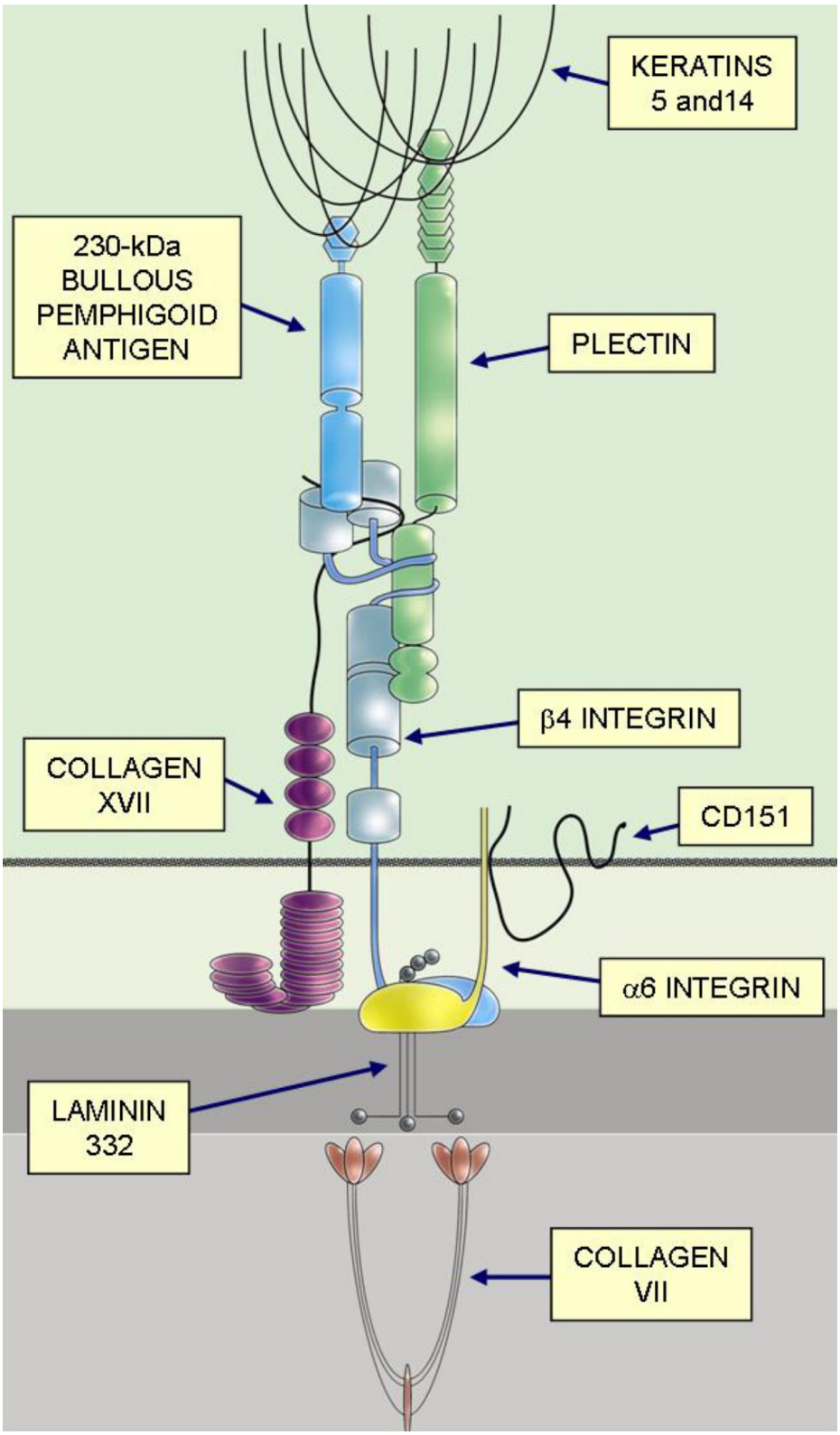


Figure 1.3 Diagrammatic representation of the macromolecular components of the basement membrane zone. The protein subdomains of the basement membrane components are shown as well as their intermolecular interactions. (Courtesy of Prof. John McGrath).



Keratin intermediate filaments within the basal keratinocytes are linked to the inner hemidesmosomal plaque via the bullous pemphigoid antigen 1 (BPAG1, BP230) and plectin (Wiche *et al.*, 1984; Guo *et al.*, 1995). On the other hand, the outer hemidesmosomal plaque via the transmembrane proteins bullous pemphigoid antigen 2 (BPAG2, BP180) and $\alpha_6\beta_4$ integrin, connects to the underlying anchoring filaments within the lamina lucida of the basement membrane (Sonnenberg *et al.*, 1991; Masunaga *et al.*, 1996; Giudice *et al.*, 1992).

The anchoring filaments are composed of the laminin-332 protein (Aumailley *et al.*, 2003) that aids in fortifying the BMZ by associating with the type IV collagen of the lamina densa (Xia *et al.*, 1996; Mayer *et al.*, 1998). In turn, the anchoring fibrils composed of collagen VII, extend from the lamina densa into the papillary dermis (Sakai *et al.*, 1986). These structures are thought to attach, via the non-collagenous-1 domain (NC1), to collagens I, III, and IV, fibronectin and laminin-332, further augmenting dermal-epidermal adhesion (Brittingham *et al.*, 2006; Wegener *et al.*, 2013).

1.3 EB classification and patterns of inheritance

The integrity of the BMZ is dependent on the functional preservation of its various components. Any disruption in the macromolecular network at the dermal epidermal junction (DEJ) can lead to various planes of skin cleavage with resultant cutaneous fragility (Fine *et al.*, 2014) (Figure 1.4). The associated clinical features are equally distinct. The plane of cleavage within the skin, coupled to other ultrastructural, immunohistochemical, molecular and phenotypic findings, determine the various subtypes of EB: EB simplex (EBS),

junctional EB (JEB), dystrophic EB (DEB) and the more recently included Kindler syndrome (Fine *et al.*, 2014). Rigorous scrutiny of the clinical findings in EB and the identification of novel effector genes has led to an evolving classification that has seen the elimination of various eponyms and the introduction of new clinical variants (Table 1.1). In the latest 2014 classification of EB (Fine *et al.*, 2014), some terms that have been used in recent studies have been made redundant (for example, RDEB-other has been substituted with RDEB- generalised intermediate). However, in citing these studies I have chosen to adhere to the nomenclature as described at that time and for consistency, the old names have been retained in this thesis.

Figure 1.4 A schematic outlining the cleavage planes in the main subtypes of EB. Three general subtypes are defined based on the plane of cleavage within the basement membrane zone. The newly added subtype; Kindler syndrome, may have variable planes of tissue cleavage.

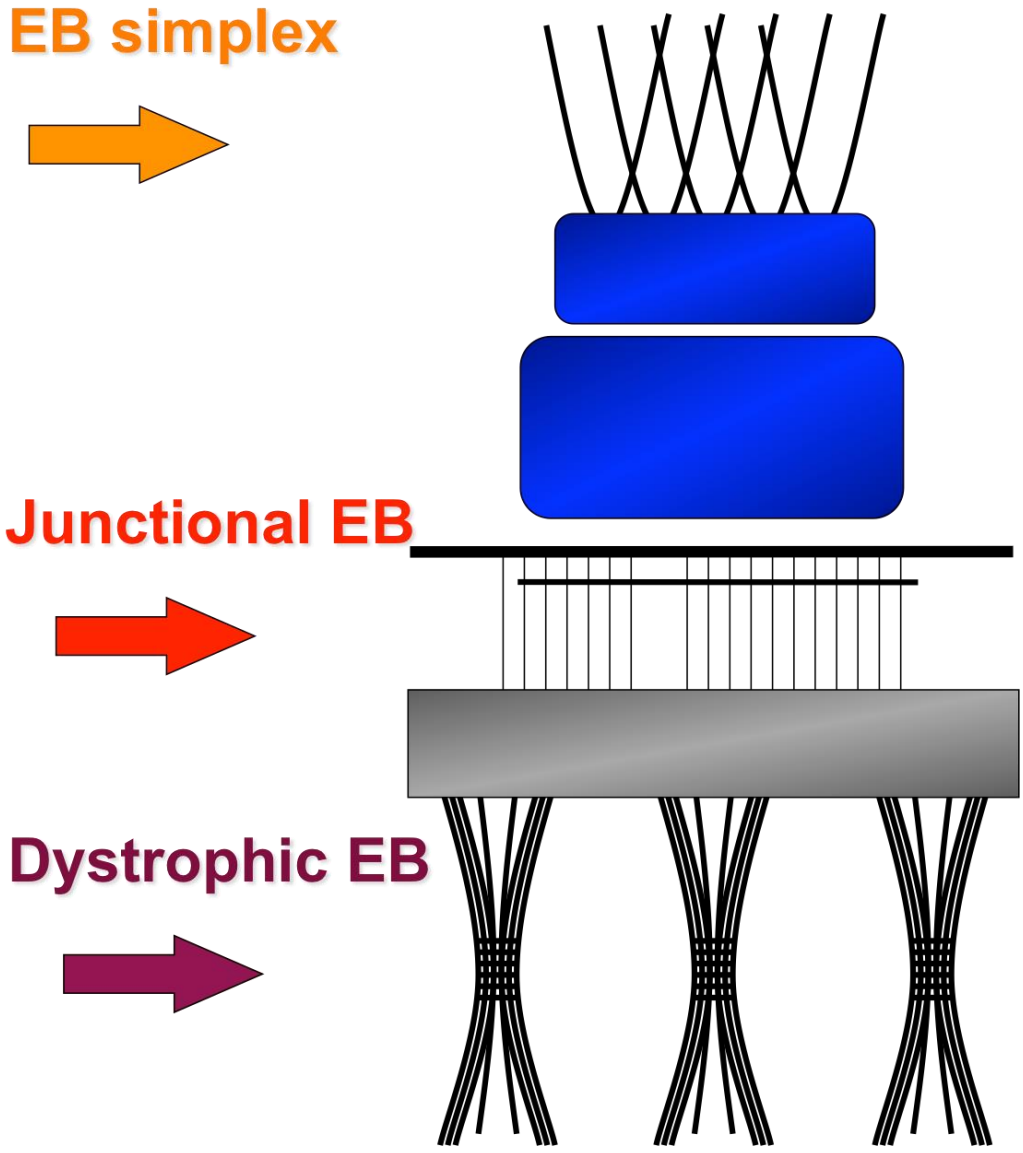


Table 1.1 The 2014 classification of epidermolysis bullosa.

Major EB types	Major EB subtypes	Subtypes	OMIM	Affected gene	Gene symbol
EBS	Suprabasal	EBS superficialis (EBSS)	607600	unknown	unknown
		Acral peeling skin syndrome (APSS)	609796	Transglutaminase 5	*TGM5*
		Acantholytic EBS (EBS-acanth)	609638	Desmoplakin, plakoglobin	*DSP, JUP*
		EBS, plakophilin deficiency		plakophilin-1	*PKP1*
		EBS, plakoglobin deficiency		plakoglobin	*JUP*
		EBS, desmoplakin deficiency		desmoplakin	*DSP*
	Basal	EBS, localised (EBS-loc)	131800	keratin 5, keratin 14	*KRT5, KRT14*
		EBS, muscular dystrophy (EBS-MD)	226670	plectin	*PLEC1*
		EBS, autosomal recessive K14 (EBS-AR K14)	601001	keratin 14	*KRT14*
		EBS, autosomal recessive- BP230 deficiency (EBS-AR BP230)		bullous pemphigoid antigen-1	*DST*
		EBS, autosomal recessive- exophilin 5 deficiency (EBS-AR exophilin 5)		exophilin 5	*EXPH5*
		EBS with pyloric atresia (EBS-PA)	612138	plectin; α6β4 integrin	*PLEC1, ITGA6, ITGB4*
		EBS with mottled pigmentation (EBS-MP)	131960	keratin-5	*KRT5*
		EBS, generalised severe (EBS-gen sev)	131900	keratin 5, keratin 14	*KRT5, KRT14*
		EBS, generalised intermediate (EBS-gen intermed)	131900	keratin 5, keratin 14	*KRT5, KRT14*
		EBS, Ogna (EBS-og)	131950	plectin	*PLEC1*

		EBS, migratory circinate (EBS-migr)	609352	keratin 5	*KRT5*
JEB	Generalised	JEB, generalised severe (JEB-gen sev)	226700	laminin-332	*LAMA3, LAMB3, LAMC2*
		JEB, generalised intermediate (JEB-gen intermed)	226650	laminin-332; collagen XVII	*LAMA3, LAMB3, LAMC2, COL17A1*
		JEB with respiratory and renal involvement (JEB-RR)		Integrin α3 subunit	*ITGA3*
		JEB, pyloric atresia (JEB-pa)	226730	α6β4 Integrin	*ITGA6, ITGB4*
		JEB, late onset (JEB-LO)	226440	collagen XVII	*COL17A1*
	Localised	JEB, localised (JEB-loc)	226650	collagen XVII, α6β4 Integrin, laminin-332	*COL17A1, ITGB4, LAMA3, LAMB3, LAMC2*
		JEB-LOC syndrome (laryngo-onycho-cutaneous)	245660	laminin-332 α3 subunit	*LAMA3A*
		JEB, inversa (JEB-i)	226650	laminin-332	*LAMA3, LAMB3, LAMC2*
DEB	Dominant	DDEB, generalised (DDEB-gen)		collagen VII	*COL7A1*
		DDEB, bullous dermolysis of the newborn (DDEB-BDN)	131705	collagen VII	*COL7A1*
		DDEB, pruriginosa (DDEB-pr)	604129	collagen VII	*COL7A1*
		DDEB, acral (DDEB-ac)		collagen VII	*COL7A1*
		DDEB-nails only (DDEB-na)	607523	collagen VII	*COL7A1*
		DDEB, pretibial (DDEB-pt)	131850	collagen VII	*COL7A1*
	Recessive	RDEB, generalised severe (RDEB-gen sev)	226600	collagen VII	*COL7A1*
		RDEB, generalised intermediate (RDEB-gen intermed)*		collagen VII	*COL7A1*
		RDEB, localised (RDEB-loc)	226600	collagen VII	*COL7A1*
		RDEB, bullous dermolysis of the newborn (RDEB-BDN)	131705	collagen VII	*COL7A1*
		RDEB, pruriginosa (RDEB-pr)	604129	collagen VII	*COL7A1*

		RDEB, inversa (RDEB-i)	226600	collagen VII	*COL7A1*
		RDEB, pretibial (RDEB-pt)	131850	collagen VII	*COL7A1*
		RDEB, centripetalis (RDEB-ce)		collagen VII	*COL7A1*
Kindler syndrome	Kindler	Kindler	173650	fermitin family homologue 1 (kindlin-1)	*FERMT1 (KIND1)*
Adapted from the 2014 EB classification (Fine *et al.*, 2014). *Previously known as RDEB-other (RDEB-O).					

In EBS the plane of cleavage is usually at the level of the basal keratinocytes as a result of dominant mutations in keratin 5 or 14 (*KRT5*, *KRT14*), plakophilin-1 (*PKP1*), desmoplakin (*DSP*), plakoglobin (*JUP*), plectin (*PLEC1*), transglutaminase 5 (*TGM5*) or α6β4 integrin (*ITGA6/ITGB4*) (Fine *et al.*, 2014). More recently, two new recessive forms have been identified secondary to mutations in the dystonin gene (*DST*) that encodes the coiled-coil domain of the bullous pemphigoid antigen 1-e (BP230) isoform (Groves *et al.*, 2010) and with mutations in *EXPH5* (exophilin-5, also known as Slac2-b, a Rab-GTPAse effector protein) (McGrath *et al.*, 2012).

JEB results in separation at the level of the lamina lucida secondary to recessive mutations in the laminin-332 (*LAMA3/LAMB3/LAMC2*), collagen XVII (*COL17A1*) or α6β4 integrin (*ITGA6/ITGB4*) genes (Fine *et al.*, 2014).

Kindler syndrome, a relatively recent addition to the EB classification, involves loss of function mutations in the actin cytoskeleton-associated protein, fermitin family homologue 1 (*FERMT1*), resulting in separation at the basal keratinocyte layer (Fine *et al.*, 2014).

All subtypes of DEB, the main focus of this thesis, result from mutations in the collagen VII gene, *COL7A1* (Christiano *et al.*, 1997b; Fine *et al.*, 2014). Collagen VII is the major component of anchoring fibrils at the DEJ and in DEB the anchoring fibrils show variable numerical and/or structural abnormalities, leading to blistering beneath the lamina densa.

In dominant forms of DEB (DDEB), the pathogenic mutations typically involve heterozygous (dominant-negative) glycine substitutions (GS) within the collagen VII triple helix resulting in a relatively mild phenotype (Uitto *et al.*, 1994; Whittock *et al.*, 1999; Dang and Murrell, 2008). In contrast, the

molecular pathology of recessive DEB (RDEB) usually comprises nonsense, frameshift or splice site mutations on both *COL7A1* alleles, which lead, in the more severe cases, to mutilating disease and early demise (Uitto *et al.*, 1994; Whittock *et al.*, 1999; Dang and Murrell, 2008).

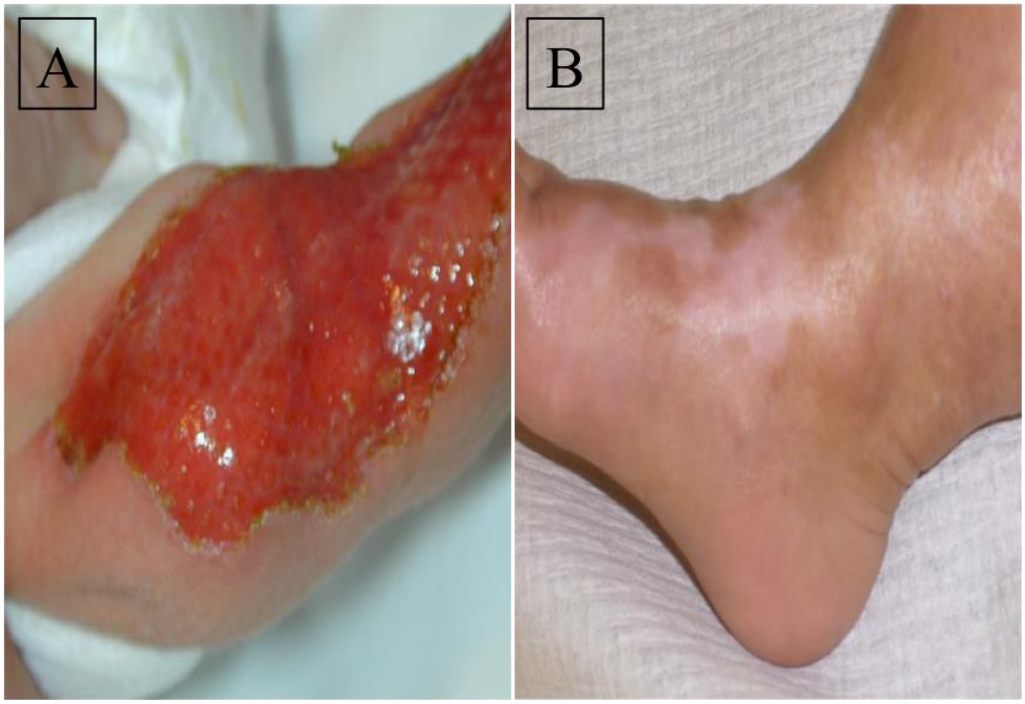
1.4 Clinical manifestations of DEB

The genetic heterogeneity underlying DEB reflects the variable phenotypic features encountered in DEB subtypes. In DDEB patients usually present with mild but varying degrees of cutaneous and mucous membrane blistering, as well as nail dystrophy (Figure 1.5). Two distinct subtypes of DDEB include EB pruriginosa (EB-pr), and bullous dermolysis of the newborn (BDN). In EB-pr, an itchy variant of DDEB (and RDEB in some cases), the pruritus can result in skin changes resembling acquired disorders such as nodular prurigo or hypertrophic lichen planus (Figure 1.6). However, in BDN another subtype of DDEB and RDEB, the blistering usually improves markedly or even regresses completely within the first year of life (Figure 1.7).

Figure 1.5 Clinical features of dominant dystrophic EB. A 10-year-old girl has evidence of mild blistering and scarring at areas of trauma, in addition to nail dystrophy.

Figure 1.6 Clinical features of EB pruriginosa (EB-pr). A 45-year-old male of Pakistani origin with evidence of excoriations on both shins with scarring and prurigo-like lesions.

Figure 1.7 Clinical features of bullous dermolysis of the newborn (BDN). (A) A 1-month-old infant with evidence of skin fragility, which was present at birth (shown here on the right inner leg). (B) Skin blistering settled by the first year of life leaving areas of hypopigmentation (shown here on the right foot).



RDEB on the other hand, usually results in a more severe phenotype. The severe generalised from of RDEB; RDEB-sev gen (previously known as Hallopeau-Siemens RDEB) results in generalized blistering culminating in mutilating scars, with subsequent joint contractures and fusion of the digits (Figure 1.8). Adding to the morbidity are extracutaneous manifestations affecting the gastrointestinal, urological and ophthalmic systems amongst others (Figure 1.9). This eventually leads to squamous cell carcinomas (Figure 1.10), a leading cause of mortality in RDEB patients and thought to result in the death of 55% of severe RDEB patients by the age of 40 years (Fine, 2009a; Fine *et al.*, 2009b; Fine and Mellerio, 2009c).

Other subtypes of RDEB include the less severe forms; RDEB-other (encompasses the newly classified entities; RDEB- generalised intermediate and RDEB-localised) (Figure 1.11), EB-pr and BDN.

Figure 1.8 Clinical features of severe recessive dystrophic EB (RDEB-sev gen). An 18-year-old male with evidence of widespread skin fragility and scarring (A), as well as flexion contractures of the digits (B).

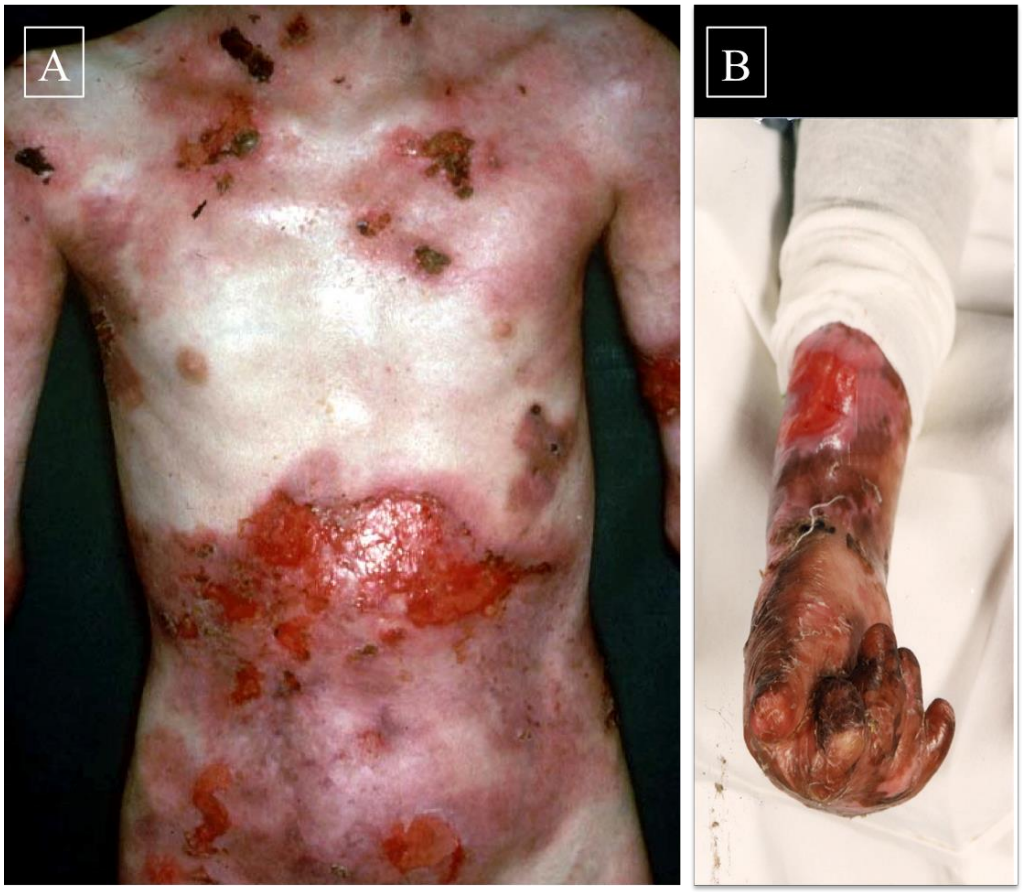


Figure 1.9 Extracutaneous features of severe RDEB. There is involvement of the oral and conjunctival mucosae with the development of synechiae (A, B), as well as oesophageal stricturing (C, arrow) secondary to blistering and subsequent scarring.

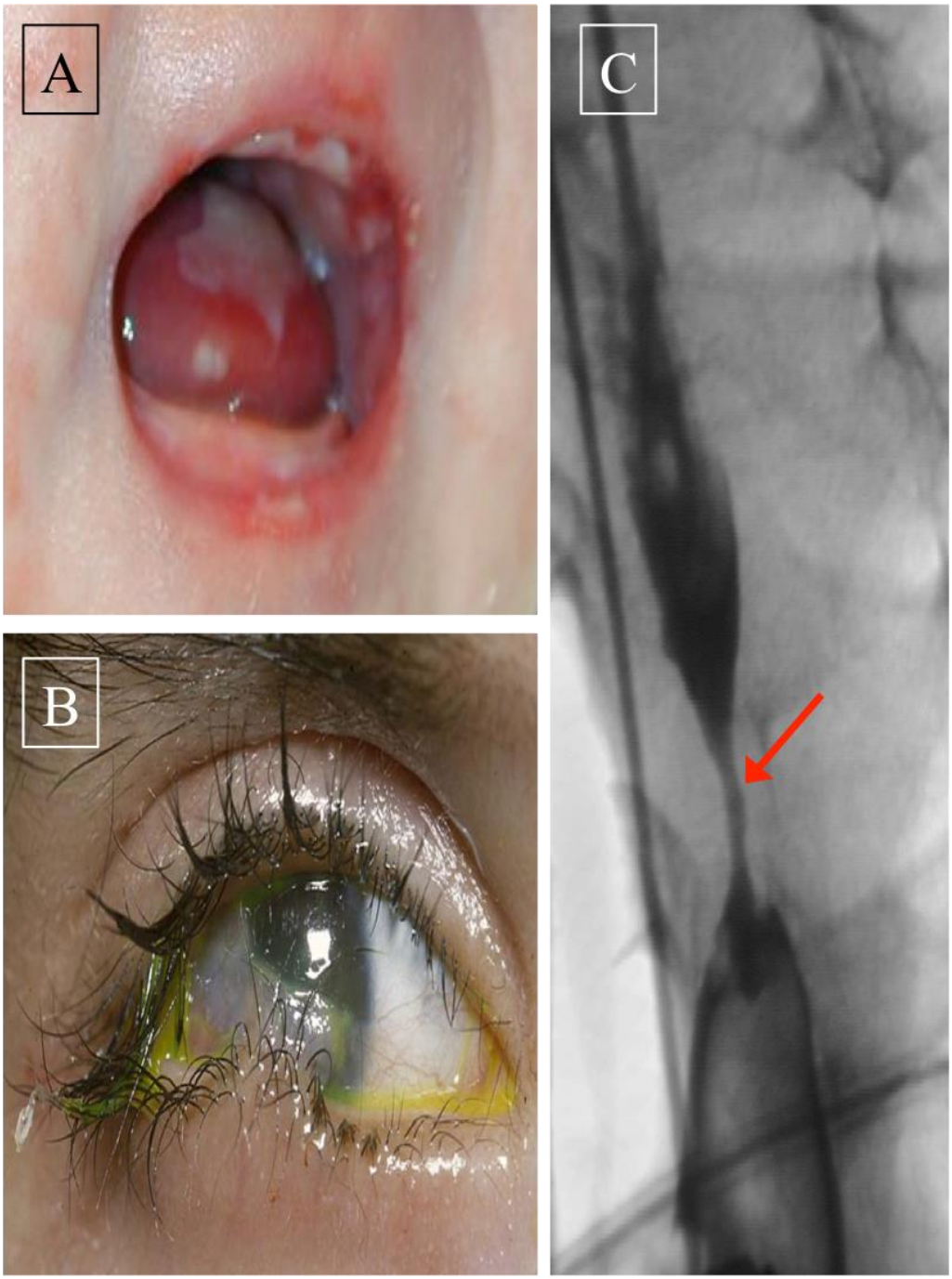


Figure 1.10 Squamous cell carcinoma complicating severe RDEB. A 28-year-old male with an advanced fungating SCC on the dorsum of the right hand. The clinical tumour margins are outlined by the dashed line.

Figure 1.11 Clinical features of mild RDEB. (A) A10-year old and (B) 2-year old girls with evidence of moderate skin fragility and scarring with milia at sites of trauma on the knees and dorsum of the right hand, respectively.

However, despite striking clinical differences, the nature of the underlying pathogenic *COL7A1* mutations in both DDEB and RDEB may overlap, and to date limited paradigms of DEB have been established (Christiano *et al.*, 1996c).

Understanding the molecular, immunohistochemical and ultrastructural alterations in DEB including the nature, type and location of *COL7A1* mutations, as well as potential genetic and non-genetic modifiers is crucial in attempting to establish a better genotype-phenotype correlation in EB.

1.5 Immunohistochemical and ultrastructural abnormalities in DEB

The plane of cleavage within the skin, as well as the degree of collagen VII expression can be established by immunofluorescence labelling of collagen VII along the basement membrane, as well as by direct visualisation of the anchoring fibril number and morphology on transmission electron microscopy (TEM). The level of abnormality in most cases, mirrors the degree of clinical severity. The LH7.2 monoclonal anti- collagen VII antibody is often used for direct immunofluorescence (DIF) and targets the non-collagenous, NC1 domain of collagen VII. This usually reveals near normal, reduced or absent labelling at the DEJ in DDEB, mild RDEB and RDEB-sev gen, respectively (Heagerty *et al.*, 1986; McGrath *et al.*, 1993a) (Figure 1.12). Labelling of other basement membrane components in patients with DEB such as type IV

collagen, laminin-332, β4 integrin subunit and collagen XVII is usually normal (Bruckner-Tuderman *et al.*, 1988).

The immunofluorescence findings in DEB are mirrored by ultrastructural changes on TEM. Patients with RDEB usually have complete absence or significant reduction in anchoring fibril numbers with only occasional wisp-like structures, whereas patients with DDEB have anchoring fibrils of near-normal morphology but reduced density (McGrath *et al.*, 1993b) (Figure 1.13).

Moreover, some subtypes of DEB have unique ultrastructural and immunohistochemical features. In BDN there is variable reduction in collagen VII labelling at the DEJ, as well as characteristic intracytoplasmic deposits of collagen VII within basal and suprabasal keratinocytes (Hashimoto *et al.*, 1985; Hashimoto *et al.*, 1989; Fine *et al.*, 1990). Ultrastructurally, there may be sub-lamina densa blistering with reduced or absent anchoring fibrils, in combination with dilated rough endoplasmic reticulum and intracytoplasmic stellate bodies. In Kindler syndrome, TEM typically reveals reduplication of the lamina densa but normal hemidesmosomes and anchoring fibrils

Figure 1.12 Immunofluorescence findings in RDEB. Basement membrane labelling with the LH7.2 monoclonal anti-type VII collagen antibody reveals bright linear labelling in normal skin (A) but complete absence in severe RDEB (B, arrows). RDEB sev gen- recessive dystrophic epidermolysis bullosa- severe generalised. Bars = 50 μm.

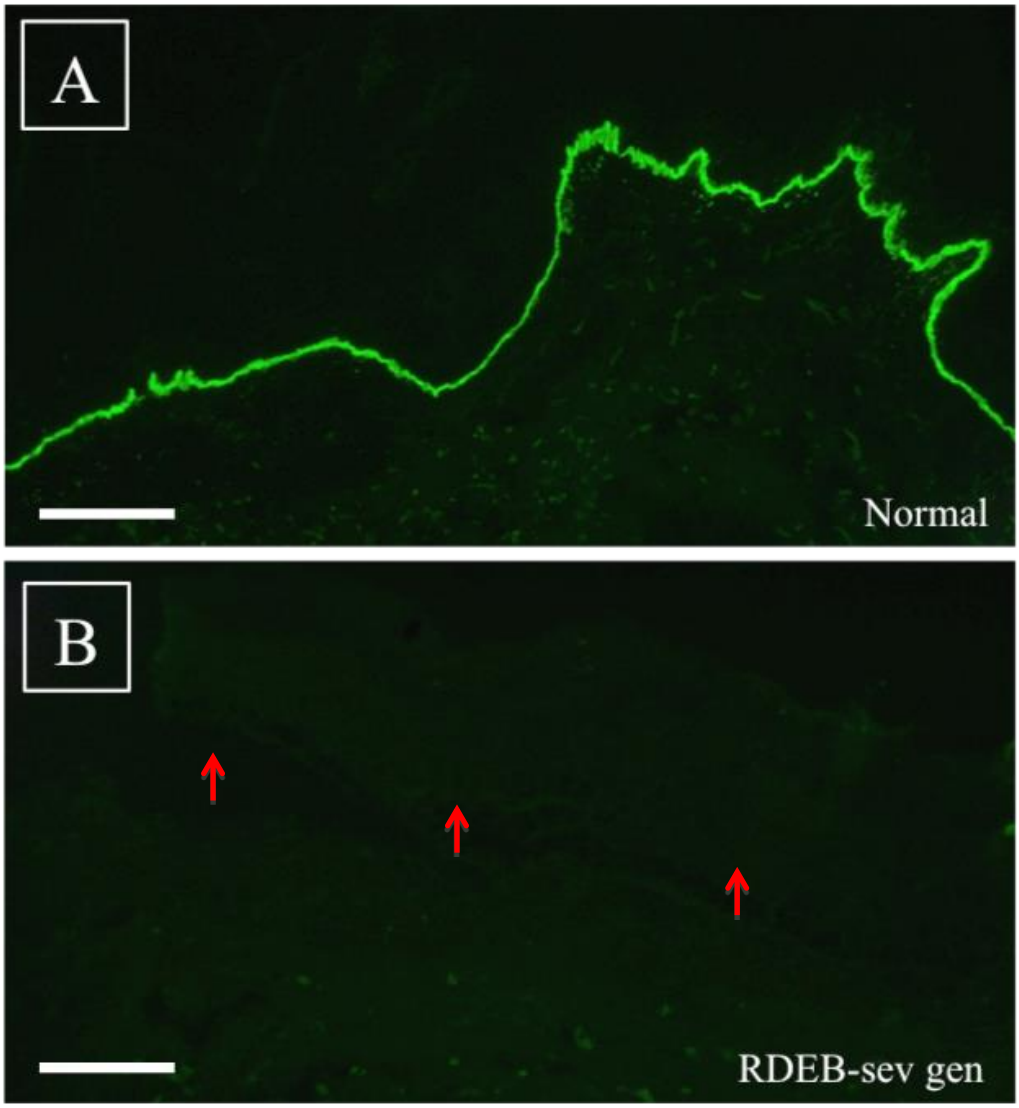
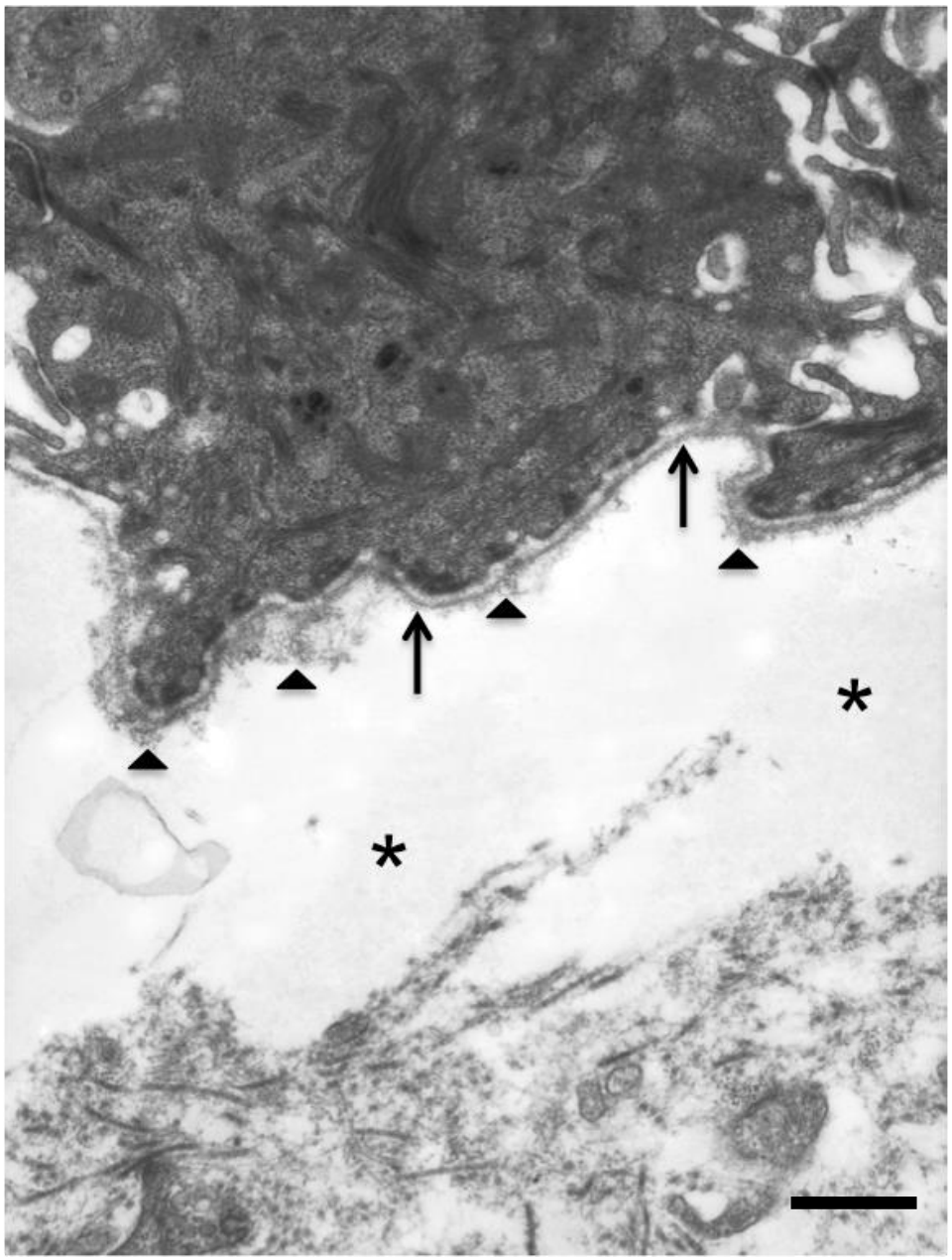


Figure 1.13 Transmission electron microscopy findings in RDEB. The dermal-epidermal separation (asterix) is shown below the level of lamina densa (arrows). Only wisp-like rudimentary anchoring fibrils are visible (arrow heads) and these are partially obscured by fibrin. Bars = 2.5 μm.



1.6 Collagen VII overview

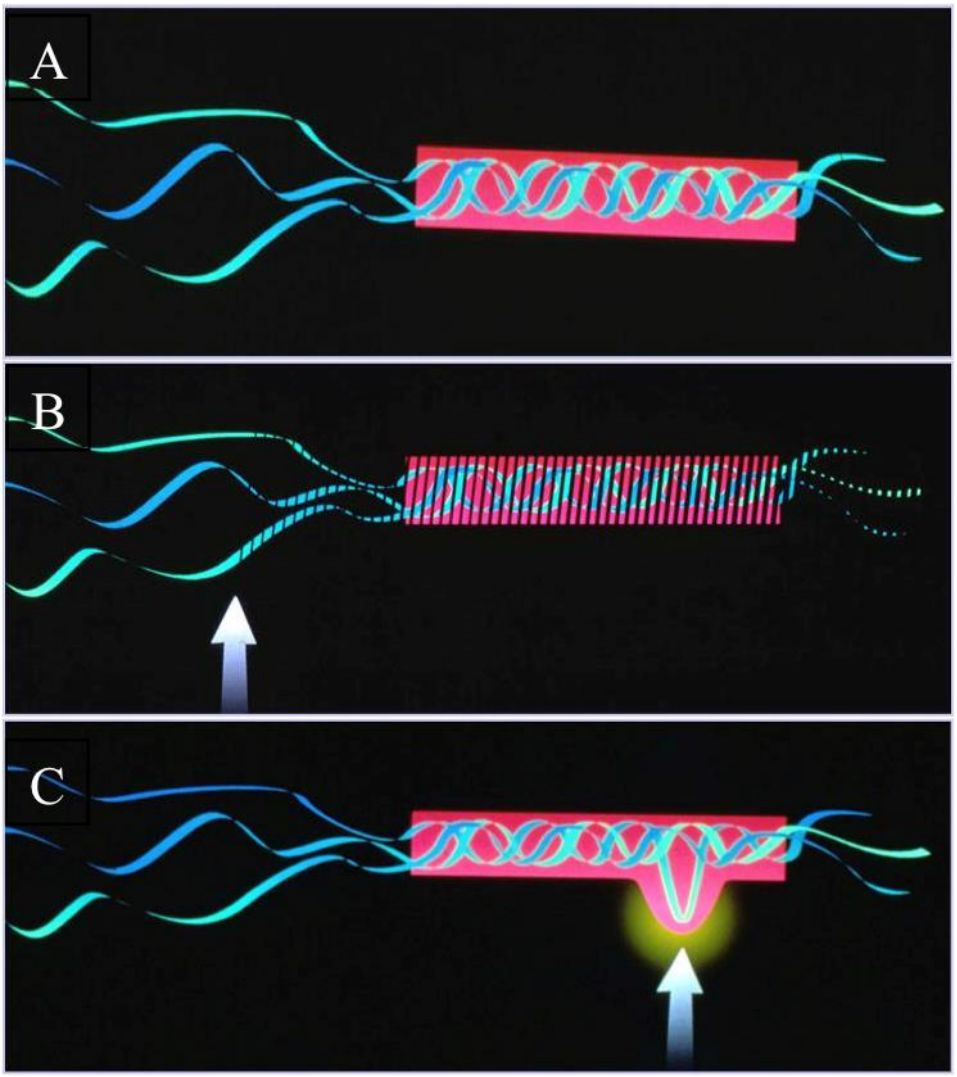
Collagens are divided into 2 major types; fibrillar and non-fibrillar collagens based on their suprastructural ability to form macromolecular fibrils. The fibrillar collagens are fibre-forming collagens with a highly conserved genetic makeup (Boot-Handford and Tuckwell, 2003; Myllyharju and Kivirikko, 2004; Prockop and Kivirikko, 1995). They are composed of monofibril, glycine-rich triple helices with uninterrupted Gly-*X*-*Y* repeats and predictable exon lengths. Non-fibrillar collagens on the other hand, are more heterogeneous. They contain multiple interruptions to the Gly-*X*-*Y* repeats and show a less well-conserved genetic makeup. Both groups however, share a triple helical structure that is formed of 3 α-polypeptides, in addition to a high glycine index with repeated Gly-*X*-*Y* sequence. Glycine, the smallest of the amino acids, is usually centred within collagen triple helices, whereas the *X*-*Y* residues are positioned exteriorly (Persikov *et al.*, 2004; Fritsch *et al.*, 2009). As a consequence, glycine amino acids are integral to triple helical stability. Any disruption or substitution with a bulkier amino acid is likely to result in abnormal suprastructural conformation and subsequent clinical pathology (Prockop and Kivirikko, 1995; Myllyharju and Kivirikko, 2001; Myllyharju and Kivirikko, 2004; Boot-Handford and Tuckwell, 2003; Fritsch *et al.*, 2009; Persikov *et al.*, 2004).

Collagen VII is a member of the non-fibrillar collagens characterised by high glycine content. The collagen VII gene (*COL7A1*) is a complex and unusually compact gene located on chromosome 3p21 (Ryynanen *et al.*, 1991; Parente *et al.*, 1991). It consists of 118 exons spanning 32kb and is transcribed

into a 9.2-kb mRNA transcript, among the largest DNA:mRNA ratio of any gene (Christiano *et al.,* 1994b; Christiano *et al.,* 1994c). Collagen VII molecules are homotrimers, composed of 3 identical α1 chains (Figure 1.14). Each chain contains a central 145-kD collagenous domain, which is flanked by non-collagenous regions; the NC1 amino and NC2 carboxy domains. The NC1 domain consists of various sub-modules with homology to adhesive proteins; cartilage matrix proteins (CMP), fibronectin-III domain, von-willebrand factor A-like motif and a proline and cysteine rich domain (Christiano *et al.,* 1994c; Burgeson *et al.*, 1990). The central collagenous domain contains 19 non-collagenous interruptions, the largest of which measures 39aa. This area, termed the 'hinge region' is thought to confer flexibility necessary for collagen VII suprastructural folding (Christiano *et al.,* 1994c; Christiano *et al.,* 1994b).

The 20-kDa NC2 domain (exon 112-118) contains conserved cysteine residues that are important for the formation of disulphide bonds within the collagen VII triple helix and antiparallel dimer assembly during fibrillogenesis (Burgeson, 1993; Greenspan *et al.,* 1993; Christiano *et al.,* 1993; Chen *et al.,* 2001). It is subsequently proteolytically removed by bone morphogenetic protein-1 (Rattenholl *et al.,* 2002). The function of this domain has not been fully elucidated but it was found to have a segment with homology to the Kunitz protease inhibitor (Greenspan *et al.,* 1993), which was postulated to result upon cleavage in negative feedback towards tissue proteinases (Bruckner-Tuderman *et al.,* 1995).

Figure 1.14 A schematic of the collagen VII homotrimers. (A) 3 identical α1 chains form a triple helical structure. Abnormality in any of the chains will lead to disruption to the collagen VII suprastructure. (B) A nonsense mutation results in a truncated protein whereas a missense mutation (C) usually results in full-length but unstable protein. (Courtesy of Prof. John McGrath).



Collagen VII, which is produced from keratinocytes and fibroblasts to a lesser extent (Ryynanen *et al.*, 1991; Chen *et al.*, 1994; Stanley *et al.*, 1985) is secreted from the intracellular space as pro-collagen homotrimers. The homotrimers then undergo processing to become collagen VII before incorporation into anchoring fibrils found in the stratified squamous epithelium of the skin and mucous membranes. These pro-collagen homotrimers initially form antiparallel dimers, which are secured by disulphide bonds at the carboxy termini following cleavage of the NC2 domain. These staggered structures then undergo polymerisation and ultrastructural folding to form loop-like anchoring fibrils, which attach through the NC1 termini to the basement membrane (BM) (Burgeson, 1993). By trapping fibrous dermal tissue, these 800nm anchoring fibrils help to fortify the dermal-epidermal adhesion. Therefore, disruption to any aspect of the collagen VII fibrillogenesis can lead to structural or numerical alteration of the collagen fibrils resulting in deleterious effects on dermal-epidermal adhesion. This eventually manifests in a sub-lamina densa plane of cleavage characteristic of DEB. However, despite the deleterious effects of abnormal anchoring fibrils, only low levels of wild-type collagen VII expression are required to maintain skin integrity as evident by observations that perhaps a 30-40% level of expression is adequate for skin stability (Fritsch *et al.*, 2008).

1.7 Collagen VII gene expression

The regulation of *COL7A1* expression is influenced by various cytokines including tumour necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1) and interleukin-1β (IL-1β) which were found to increase

collagen VII gene and protein expression by dermal fibroblasts in culture (Chen *et al.*, 1994; Amano *et al.*, 2007; Mauviel *et al.*, 1994). In contrast, *COL7A1* and protein expression by epidermal keratinocytes is reduced by TNF-α and IL-1β. However, in the presence of TGF-β1, these cytokines enhance TGF-β induced *COL7A1* gene and protein expression (Takeda *et al.*, 2005). TGF-β and TNF-α response elements were identified in distinct regions of the *COL7A1* promoter area and are regulated by transcription factors SMAD and NF-kappaβ (Kon *et al.*, 1999; Vindevoghel *et al.*, 1998). JUN and FOS, as part of the activator protein-1 (AP-1) transcription complex were also found to increase *COL7A1* gene expression following ultraviolet radiation through binding to AP-1 binding sites in the *COL7A1* promoter region (Nakano *et al.*, 2001).

1.8 *COL7A1* mutations

All subtypes of DEB result from mutations in the collagen VII gene, *COL7A1* (Fine *et al.*, 2014) and paradigms for genotype-phenotype correlation have emerged. In DDEB the pathogenic mutations typically involve heterozygous (dominant-negative) missense mutations and owing to the high glycine content the majority are GS mutations within the collagen VII triple helix (Uitto *et al.*, 1994; Whittock *et al.*, 1999; Dang and Murrell, 2008). The resulting macromolecular and functional consequences are variable and include effects on helix formation, protein folding, thermal stability, intracellular transport, secretion, and assembly into anti-parallel dimers. However, although only 1.6% of anchoring fibrils are wholly formed of wild type trimeric molecules, the phenotype is typically mild as despite the presence of a mutant

allele, full-length polypeptides and anchoring fibrils are formed (Kon *et al.*, 1997b).

In contrast, the molecular pathology of RDEB generally comprises nonsense, frameshift or splice site mutations on both *COL7A1* alleles (Uitto *et al.*, 1994; Whittock *et al.*, 1999; Dang and Murrell, 2008). The severe form of recessive DEB; RDEB-sev gen, usually results from homozygous or compound heterozygous loss of function mutations leading to truncated collagen polypeptides and mRNA decay (Christiano *et al.*, 1994a). Inheritance of recessive missense mutations on one or both alleles results in a milder phenotype mirroring an attenuated immunohistochemical and ultrastructural expression of collagen VII.

However the mRNA, protein and subsequent phenotypic expression is not solely determined by the type of mutation and has to be studied in conjunction with the location of this mutation within the collagen VII gene, the effects of other confounding mutations, as well as potential genetic and epigenetic modifiers.

1.9 Genotype-phenotype heterogeneity

Although all forms of DEB result from *COL7A1* mutations, there is vast genotype-phenotype heterogeneity with both intra- and inter-familial variability. The nature of the various *COL7A1* mutations whether nonsense, missense or splice site, primarily reflect functional impairment. However, other characteristics might play a role in phenotypic expression including the position of the *COL7A1* mutations within the triple helix, particularly the proximity to the 'hinge' domain and to highly conserved cysteine regions (van

den Akker *et al.*, 2011), as well as the relation to the non-collagenous NC1 or NC2 terminals (Chen *et al.*, 2001; Woodley *et al.*, 2008). Moreover, genetic, epigenetic and environmental factors are likely to play a significant role in disease expression.

The nature and location of some mutations have been associated to some degree with particular subtypes of DEB. RDEB-inversa (RDEB-I) for example, is a unique subtype of DEB characterised by involvement of the flexures. This has been associated with mutations affecting the thermal stability of *COL7A1*, particularly arginine substitution mutations within the hydrophilic highly conserved motifs of the triple helical domain or GS mutations at the borders of the collagenous subdomains (Chiaverini *et al.*, 2010).

EB-pr another form of DEB characterised by itchy prurigo-like lesions on the limbs has been associated with GS mutations within the triple helical domain as well as splice site mutations resulting in the skipping of exon 73 (Fine *et al.*, 2014; McGrath *et al.*, 1994).

BDN is characterised by skin fragility at birth that ameliorates or ceases completely within the first year of life. This subtype has been typically associated with GS substitutions affecting secretion of collagen VII leading to intracytoplasmic collections of collagen VII (Fine *et al.*, 2014; Hashimoto *et al.*, 1985).

In addition, some recurrent mutations or 'hotspots' have been identified in specific populations such as the p.Arg578Stop, c.7786delG and p.Arg2814Stop mutations in British families (Mellerio *et al.*, 1997; Mohammedi *et al.*, 1999); c.2470insG in Mexican families (Salas-Alanis *et al.*, 2000; Salas-Alanis and McGrath, 2006); c.5818delC, c.6573+1G→C and

Glu2857Stop in Japanese families (Tamai *et al.*, 1999), p.Gly1664Ala in Italian families (Gardella *et al.*, 2002), as well as the mutation p.G2043Arg in exon 73 and c.425A→G worldwide (Murata *et al.*, 2004; Mellerio *et al.*, 1998).

Moreover, there appears to be a clustering of mutations, in particular GS mutations, within exon 73 however, the nature of these pathogenic mutations in both DDEB and RDEB, is similar, and to date no definite correlation has been found between the type or location of the mutations and the resulting subtype (Christiano *et al.*, 1996c).

1.9.1 Mutations resulting in variable expression: the intra- and inter-familial phenotypic heterogeneity

Of interest is the degree of phenotypic heterogeneity associated with an identical mutation. This is not only described among unrelated individuals but also within same pedigrees, where for example a glycine substitution mutation can result in mild skin blistering, nail dystrophy only, variable subtypes of DEB or no clinical pathology. This could be explained by the presence of a yet unidentified effector mutation or incomplete penetrance. However, metabolic, genetic, epigenetic, or environmental factors are likely to have a role in modulating disease expression.

1.9.2 Mutations resulting in variable inheritance

Adding to the dilemmas of phenotypic heterogeneity are reports of variable genetic inheritance even within same families. Missense mutations, namely GS substitution mutations, are typically associated with DDEB leading to a dominant negative effect. GS mutations are also associated with recessive

DEB to a lesser degree and these are typically silent when heterozygous but pathogenic when inherited on both alleles or *in trans* with another loss-of-function mutation in *COL7A1*. However, some GS mutations result in both dominant and recessive DEB adding to the diagnostic dilemmas. Although, it is not surprising that GS mutations account for the vast majority of mutations in the *COL7A1* and collagen genes in general, owing to their high glycine content, the presence of both dominant and recessive GS mutations in the same gene/protein is unusual. The factors influencing the varied inheritance patterns are yet to be determined but could reflect confounding genetic or epigenetic factors.

1.9.3 Revertant mosaicism: individual genotypic and phenotypic heterogeneity

Revertant mosaicism (RM) refers to the occurrence of two genetically distinct populations of cells due to a spontaneous genetic correction that occurs during mitosis (Hall, 1988; Jonkman *et al.*, 1997). It has been described in various inherited conditions including severe combined immunodeficiency, Bloom's syndrome, Fanconi's anaemia, X-linked Wiscott–Aldrich syndrome, Duchenne muscular dystrophy and tyrosinemia type I (Hirschhorn, 2003). It has also been described in non-Herlitz JEB and EBS, as a result of *in-vivo* revertant mutations in *LAMB3*, *COL17A1* and *KRT14* (Darling *et al.*, 1999; Schuilenga-Hut *et al.*, 2002; Smith *et al.*, 2004; Pasmooij *et al.*, 2005; Pasmooij *et al.*, 2007; Jonkman and Pasmooij, 2009). This phenomenon is thought to be under recognised and perhaps occurs in all cases of generalised intermediate JEB (Jonkman and Pasmooij, 2009; Pasmooij *et al.*, 2012).

Proposed corrective mechanisms include back mutations, intragenic crossovers, mitotic gene conversions, and second-site mutations (Pasmooij *et al.*, 2005; Jonkman *et al.*, 1997; Frank and Happle, 2007). This phenomenon of natural gene repair is likely to be over looked in patients with DEB as well. If proven, then RM in individuals with RDEB could have significant implications for future gene and cell therapy.

1.9.4 The role of modifiers in genotype-phenotype correlation

Several studies have explored the role of metabolic, genetic, epigenetic, or environmental factors in modulating disease expression and severity. Understanding factors contributing to genotype expression will not only refine genotype-phenotype correlation but will have significant therapeutic implications.

Metabolic factors influencing pruritus have been explored in EB-pr in an effort to explain this distinctive pruritic subtype. These include biochemical and endocrinological abnormalities, iron deficiency, IgE, presence of atopy and filaggrin mutations. However, none of these have been found to directly influence disease expression (Mellerio *et al.*, 1997; Drera *et al.*, 2006; Schumann *et al.*, 2008; Ee *et al.*, 2007; Lapinski *et al.*, 1998; Ren *et al.*, 2008).

The degree of skin fragility can also be influenced by epigenetic mechanisms affecting the level of protein expression in the BMZ including the variable degradation of intracellularly accumulated mutant proteins, as well as the effects of endoplasmic reticulum stress and unfolded protein on apoptosis and gene expression (Bateman *et al.*, 2009). In a recent *in vitro* study by Knaup *et al.*, the gene expression profiles of EBS-MD, RDEB and a subtype of JEB

previously called generalized atrophic benign epidermolysis bullosa were analysed (Knaup *et al.*, 2012). All revealed up-regulation of *COL16A1* and *FN1* (fibronectin gene); in addition to accumulation of cholesterol intracellularly, factors known to confer greater resistance towards mechanical forces and stabilisation of the cell membrane respectively. In addition there was down-regulation of ribosomal protein S27A, a ubiquitin fusion protein, which could potentially result in increased degradation of mutant alleles by ubiquitin (Knaup *et al.*, 2012; Redman and Rechsteiner, 1989).

At a transcriptional level, various modulators of collagen VII expression have been studied including the variable splicing of transcriptional RNA (Nissim-Rafinia and Kerem, 2005) where some splice site mutations result in the production of alternatively spliced isomers and a variable level of functional proteins with subsequent clinical effects. Another novel mechanism involves translationally silent mutations in exonic splicing enhancer (ESE) sequences that are capable of causing exon skipping/exon definition. These sequences are capable of selecting splicing sites in a cascade that starts with binding of ESE to members of the serine⁄arginine-rich protein family and culminating in recruitment of spliceosomes and exon skipping (Covaciu *et al.*, 2011). An example is the c.2470insG mutation in *COL7A1* that results in skipping of exon 19 with a subsequent downstream premature termination codon in exon 20 (Salas-Alanis *et al.*, 1998; McGrath *et al*., 1999). Therefore, mutations in previously unrecognised ESE sequences could also modulate *COL7A1* expression. Similarly, undetected *COL7A1* mutations including large genomic deletions could also contribute to variable disease expression (Kern *et al.*, 2009a).

Moreover, mutations in genes other than *COL7A1* are likely to be involved in disease modification. In a recent study, a single nucleotide polymorphism (1G/2G) in the matrix metalloproteinase-1 (*MMP-1*) gene promoter was implicated in a more severe RDEB phenotype. MMP-1, a zinc metallo-endopeptidase capable of degrading extracellular proteins, was found to be transcriptionally upregulated, resulting in increased degradation of collagen VII with subsequent severe disease (Titeux *et al.*, 2008).

In the future, particularly with the introduction of whole genome and exome sequencing as well as data mining studies, previously unrecognised modifiers are likely to be revealed (Reverter *et al.*, 2008).

1.10 Therapeutic Options at the time of this study

To date no definitive treatment is available for EB and management is conservative aimed at preventing infection and trauma, wound care, pain management and early detection of squamous cell carcinoma. This has led to a high burden of morbidity in almost all affected individuals. The median survival in milder forms of RDEB ranges between 55-60 years (Fine, 2009a), whereas there is an increased rate of mortality in severe RDEB cases secondary to metastatic squamous cell carcinomas (SCCs), which usually leads to early demise by the 4th decade of life in around 50% of cases (Fine *et al.* 1999).

So far, understanding of the molecular and ultrastructural pathologies underlying EB has led to advances in diagnostic applications such as pre-implantation and pre-natal genetic DNA testing utilising chorionic villus sampling and amniocentesis, as well as *in vitro* testing of embryos (Fassihi *et al.*, 2006; Cserhalmi-Friedman *et al.*, 2000; Pfendner *et al.*, 2003) and possible

analysis of foetal DNA in maternal plasma (Uitto *et al.*, 2003; Chiu and Lo, 2003). In addition, the plethora of recent data relating to collagen VII structure and expression, ultrastructural and functional effects of various *COL7A1* mutations, potential genetic and epigenetic modifiers, as well as keratinocyte, fibroblast and stem cell biology have paved the way for exciting *in vivo* and *ex vivo* therapeutic applications utilising gene, protein and cell based therapies.

1.10.1 Protein based therapy

This mode of therapy utilises recombinant proteins in topical, injectable or systemic forms for the replacement of defective or absent molecular proteins. Intradermal injection of recombinant collagen VII into immunocompromised mice with DEB xenografts was attempted initially resulting in increased expression of collagen VII along the basement membrane with reduced skin fragility (Woodley *et al.*, 2004a; Woodley *et al.*, 2004b).

1.10.2 Cell therapy

The understanding of the molecular genetics underlying DEB has provided a platform for the delivery of cell-based therapy utilising keratinocytes and fibroblasts.

At the time this thesis began, cultured keratinocyte allografts, then combined keratinocyte and fibroblast grafts were utilised in the treatment of intractable skin ulcers in RDEB patients (McGrath *et al.*, 1993b; Falabella *et al.*, 2000; Falabella *et al.*, 1999). Subsequently, a cultured allogeneic dermal substitute containing fibroblasts, hyaluronic acid and collagen was used in both

animal models and humans (Kubo and Kuroyanagi, 2004; Natsuga *et al.*, 2010; Hasegawa *et al.*, 2004). Although this topical treatment induced re-epithelialisation in the intractable skin ulcers, the level of collagen VII expression mostly remained unchanged as evidenced by TEM and IF studies. This led to optimisation in the delivery of cell therapy in the form of intradermal injection in transgenic mice (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2004b), paving the way for intradermal allogeneic fibroblasts injections in RDEB patients (Wong *et al.*, 2008; Yan and Murrell, 2010). In addition, intravenous injection of human fibroblasts was also attempted in transgenic RDEB mice following which fibroblasts were shown to home to skin with subsequent improvement in wound healing (Goto *et al.*, 2006; Woodley *et al.*, 2007; Kern *et al.*, 2009b).

1.10.3 Gene therapy

Translational applications in the form of *ex vivo* and *in vivo* gene therapy for the treatment of EB have aimed to correct defective genes in keratinocytes and fibroblasts. Viral and non-viral vectors, as well as microinjection techniques have been utilised to introduce wild-type *COL7A1* into DEB cells (Ortiz-Urda *et al.*, 2003; Mecklenbeck *et al.*, 2002). In one study, gene-corrected fibroblasts injected intradermally into RDEB organotypic skin-equivalents grafted onto immunodeficient mice resulted in restoration of collagen VII expression at the DEJ (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003). Subsequently intravenous injection of genetically engineered fibroblasts in animal models was also shown to lead to over-expression of collagen VII (Woodley *et al.*, 2007).

Genetically modified fibroblasts and keratinocytes have also been used to create bioengineered skin grafts to improve skin fragility. However this is a non-definitive localised method of treatment, which is limited by difficult gene transfer into epidermal keratinocytes, low level of maintained collagen VII expression as well as variable success of engraftment (Sat *et al.*, 2000; Ghazizadeh *et al.*, 1999). Therefore, to enhance delivery and increase bioavailability *in vivo*, gene transfer of *COL7A1* into transgenic mice was attempted. This also resulted in functional correction of *COL7A1* with sustained tissue-specific production of full-length collagen VII (Sat *et al.*, 2000). Regeneration of normal skin was also demonstrated *in vivo* by the application of lentivirus-transduced DEB keratinocytes and fibroblasts onto the skin of SCID mice (Chen *et al.*, 2002b).

More recently, genetic correction of stem cells was attempted for the treatment of JEB. Epidermal stem cells harbouring compound heterozygous *LAMB3* mutations were transduced with full-length wild-type transgene and then propagated in culture to form epithelial grafts that were transplanted onto the same patient with resultant functional correction in grafted sites (Mavilio *et al.*, 2006). In principal, this approach could provide corrected stem cells capable of differentiating into functional, mutation free cells. However, the use of some DNA-vectors has the potential for inducing carcinogenesis, thus limiting its therapeutic potential.

In addition, natural gene therapy in the form of revertant mosaicism has emerged as an exciting therapeutic resource in patients with JEB. These individuals -as a result of spontaneous genetic correction- were found to harbour 2 populations of genetically distinct cells leading to functional protein

expression with clinically normal patches of skin. This phenomenon has led to the use of naturally corrected autologous keratinocytes to create epidermal sheets or grafts that were then transplanted onto the same patients circumventing issues related to genetic transduction and carcinogenesis (Pasmooij *et al.*, 2007; Pasmooij *et al.*, 2005; Jonkman *et al.*, 1997; Darling *et al.*, 1999; Gostynski *et al.*, 2009).

1.10.4 Drug therapy

At the time of this thesis, pharmacologic therapies in the form of antimicrobials and anti-inflammatory agents had been used widely to reduce bacterial load and promote wound healing in DEB including phenytoin (Caldwell-Brown *et al.*, 1992), trimethoprim (Lara-Corrales *et al.*, 2012) and tetracyclines (Humbert *et al.*, 1989) with variable success. Many of these agents however, are thought to also possess anti-collagenase activity, which can augment wound-healing properties. Some immunosuppressant agents such as ciclosporin (Husz *et al.*, 1989), have also been used due to their immunomodulatory effect but the exact mechanism by which they ameliorate skin fragility is not understood. Furthermore, topical and systemic agents were specifically used to modulate the disabling pruritus associated with EB-pr with some effect. These include topical and systemic corticosteroids, cryotherapy, topical tacrolimus, ciclosporin and thalidomide (Yamasaki *et al.*, 1997; Ozanic Bulic *et al.*, 2005; Banky *et al.*, 2004; Das *et al.*, 2005). However, these were largely aimed at symptomatic relief rather than definitive therapy.

In conclusion, advances in molecular, immunohistochemical and ultrastructural diagnostics as well as in depth understanding of EB has led to exciting advances in protein, cell and gene therapies. However, several aspects of the genotype and phenotype heterogeneity encountered in DEB are yet to be explained, although genetic, epigenetic and environmental modulators of *COL7A1* expression have been implicated. As a consequence, the ideal therapy of DEB is likely to involve a combination of agents targeting various patient-specific aspects of DEB modulation. The refining of genotype-phenotype correlation will not only increase our understanding of EB pathology and phenotypic heterogeneity but will also enable us to identify modulators of disease expression and ultimately achieve more effective, patient specific and targeted therapies for DEB.

1.11 Hypothesis and aims of the thesis

The hypothesis of this thesis is that more detailed analysis of the molecular and cellular pathology of the skin in individuals with clinically unusual or diverse forms of DEB will help refine genotype-phenotype correlation with relevance to further improving the classification of EB.

The thesis addresses the following specific aims:

[1] To investigate the molecular pathology in *COL7A1* associated with intra-epidermal retention of collagen VII and to determine the clinical ramifications.

[2] To update and expand the spectrum of dominant and recessive glycine substitution mutations in dystrophic EB.

[3] To review and investigate the role and clinicopathologic consequences of non-glycine missense substitutions in collagen VII in both dominant and recessive forms of EB.

[4] To investigate the role of genetic modifiers on disease expression in EB.

[5] To explore the possibility of revertant mosaicism in RDEB.

[6] To investigate the role of intradermal allogeneic fibroblasts on collagen VII expression in RDEB.

Chapter 2

Materials and methods

This MD project was undertaken following ethical approval by the Guy's and St Thomas' Hospital local Ethics' Committee, reference number 07/H0802/104. Informed consent was obtained from the involved subjects.

The work included in this thesis refers to my work except where retrospective analysis of archival data was conducted. In Chapters 3, 4 and 5 the majority of the DNA mutation analyses, immunofluorescence and electron microscopy studies where performed previously in the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory in London. The studies were conducted as part of the patients' routine clinical care in most cases, where samples were sent from local, national and international medical teams. However, for individuals linked to separate research projects, studies were undertaken with Ethics' Committee approval. Patient ethnicities included white Caucasian, Middle-Eastern, South American, South-East Asian and Asian.

Clinical information was obtained from the medical records, relevant publications or by contacting the referring clinician(s) where possible. However, in many cases, particularly for historic samples, clinical data was obtained from the information supplied with the original referral forms or from the laboratory logbook. Due to the required technical expertise, electron microscopy studies were done by Patricia Dopping-Hepenstal in the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory in London.

I performed all *MMP-1* mutation analyses and statistical studies described in Chapter 6. In additional to the laboratory work, I performed the clinical work described in Chapters 7 and 8 including skin biopsies and allogeneic fibroblast injections.

2.1 Molecular Biology Techniques

2.1.1 Genomic DNA extraction from peripheral blood leukocytes

Genomic DNA was extracted from peripheral blood using the QIAmp Blood Midi Kit (Qiagen, Crawley, UK). Blood samples were initially equilibrated to room temperature. 1ml of blood was mixed briefly with 100µl of proteinase K (QIAGEN Protease). 1.2ml of lysis buffer (Buffer AL) was then added to the mixture, which was mixed thoroughly by inversion followed by vigorous shaking for 1minute. The mixture was then incubated in a 70ºC water bath for 10minutes. 1ml of 100% ethanol was added to the sample and mixed again by inversion followed by vigorous shaking for 1minute. All of the solution was then applied carefully to the QIAmp midi column within a 15ml centrifuge tube. The cap was the closed and the tube centrifuged at 3000rpm for 3minutes. The filtrate was discarded and 4mls of washing buffer (Buffer AW1) was then added to the column, which was again centrifuged for 3minutes. This step was followed by the application of 4mls of a second washing buffer (Buffer AW2) as for Buffer AW1. To dry the membranes the tubes were vacuum-dried for 15minutes. Each column was then placed in a 15ml centrifuge tube. 200µl of Buffer AE equilibrated to room temperature was added onto the membrane and the tube was incubated at room temperature for 5minutes then centrifuged for 2minutes at 5000rpm. The eluted DNA was then analysed using the Nanodrop ND1000 UV-Vis Spectrophotometer (Labtech International Ltd., Ringmer, UK) to determine DNA concentration.

2.1.2 Genomic DNA extraction from skin biopsies

DNA was extracted from skin biopsies using the QIAamp DNA Mini Kit (Qiagen). Samples were homogenised using a hand-held rotor-stator homogeniser in Buffer ATL. 20µl proteinase K was then added to the mixture then mixed by vortexing. The suspension was then incubated at 56°C with occasional shaking until the tissue is completely lysed. The tube containing the suspension was briefly centrifuged to ensure fluid in the lid is removed. To remove undesired RNA 4µl RNAse A (100mg/ml) was then added and mixed by pulse-vortexing for 15seconds followed by incubation at room temperature for 2minutes. The tube was again briefly centrifuged, then 200 µl Buffer AL was added to the sample then mixed as for RNAse A followed by incubation at 70°C for 10minutes. Following brief centrifugation 200µl of Buffer AL was added to the sample then mixed and incubated as in the previous step. 200µl of ethanol (96–100%) was then added to the sample, and mixed by pulse-vortexing for 15seconds followed by brief centrifugation in a 1.5ml microcentrifuge tube. The mixture was then applied to the QIAamp Mini spin column, which was then closed and centrifuged at 8000 rpm for 1minute. The tube containing the filtrate was now discarded and the column placed in a clean 2ml collection tube. 500µl of Buffer AW1 was then applied to the column followed by the same procedure outlined in the last step. This was followed by the addition of 500µl of Buffer AW2. The cap was closed and the tube was centrifuged at 14,000 rpm for 3minutes. The column was then placed in a clean 1.5ml microcentrifuge tube and 200µl Buffer AE was added to the column. The tube was then incubated at room temperature for 1 min, and then

centrifuged at 8000 rpm for 1minute to elute the DNA. This step was repeated again to increase DNA yield.

2.1.3 Genomic DNA and RNA extraction from cell cultures

Total RNA was extracted using the TRIzol reagent method (Invitrogen, Paisley, UK). 1ml of TRIzol reagent was added per 10cm^2 of plated monolayer culture. The suspension was then repeatedly aspirated to lyse the cells thoroughly and then allowed to stand at room temperature for 5minutes to ensure complete dissociation of the nucleoprotein complexes. 0.2ml of chloroform was added per 1ml of TRIzol reagent used. The suspension was then centrifuged at 12000rpm for 15minutes at 4°C resulting in the separation of the mixture into 3 phases; the aqueous phase containing RNA, the interphase containing DNA and the organic phase containing proteins.

For RNA isolation the aqueous phase was then carefully transferred into a new falcon tube with the addition of 0.5ml of isopropranolol per 1ml of Tri Reagent used. The suspension was then allowed to stand for 10minutes at room temperature then centrifuged at 12000rpm for 10 minutes at 4°C. This results in an RNA precipitate at the bottom and sides of the tube. The supernatant was then aspirated and the precipitate washed with 1ml of 75% ethanol. This was followed by centrifugation at 7500rpm for 5minutes at 4°C. The supernatant was then aspirated and discarded and the RNA pellet air-dried at room temperature for 10minutes. The pellet was then resuspended in 50ul of RNAse free water then stored if needed at -80°C.

For DNA isolation and following careful removal of the aqueous phase, 0.3mL of 100% ethanol per 1mL of TRIzol reagent used for the initial

homogenization was added and then mixed by inversion. Samples were then incubated at 15 to 30°C for 2-3minutes followed by centrifugation at 2,000 x g for 5minutes at 4°C. The pellet was then washed twice in 0.1M sodium citrate in 10% ethanol (1mL per 1mL of TRIzol Reagent used for the initial homogenization). On each wash the sample was incubated for 30minutes at 15 to 30°C with frequent mixing and centrifuged at 2,000 x g for 5minutes at 4°C. The DNA pellet was then suspended in 75% ethanol (1.5-2mL of 75% ethanol per 1mL TRIzol Reagent), and then incubated for 10-20minutes at 15 to 30°C with frequent mixing then centrifugation at 2,000 x g for 5 minutes at 4°C. The pellet was then air dried for 5 to 15minutes in an open tube then dissolved in 8mM NaOH. The insoluble material was removed by centrifugation at >12,000 g for 10 minutes and the supernatant containing the DNA was transferred to a new tube then stored if needed at -80°C.

2.1.4 Extraction of total RNA from skin biopsies

RNA extraction was performed using the RNeasy fibrous tissue kit (Qiagen). The samples, originally stored in RNA later (Qiagen) at -20°C were thawed at room temperature. Samples were then homogenised using a hand-held rotor-stator homogeniser in RLT lysis buffer and β-mercaptoethanol solution. The mixture was then added to the RNeasy spin column where RNA adsorbs onto the membrane. This is then washed with various washing buffers according to the manufacturer's instructions and the RNA eluted in 30µl of RNase free water (Qiagen) and stored at -80°C.

2.1.5 DNA and RNA quantification and quality control

The concentration of extracted total DNA or RNA was measured using the Nanodrop ND1000 UV-Vis Spectrophotometer (Labtech). This was assessed through the optical density at 260nm and 280nm. The ratio of light absorbance at 260nm:280nm was used to indicate the degree of purity of the DNA and RNA samples. Only samples with an ideal 260nm:280nm absorption ratio between 1.8 and 2.1 were used for subsequent experiments.

2.1.6 Reverse transcription

cDNA was synthesized from RNA using the SuperScript II RT protocol (Invitrogen). Each reaction mixture consists of 2µg of total RNA, 50ng of random primers, 1ul of 10nM dNTP mix and RNAse and DNAse free water to a total volume of 12µl. The mixture was then heated to 65°C for 5minutes then rapidly cooled on ice. A brief centrifugation was then followed by the addition of 4µl of 5X First Strand Buffer, 2µl of 0.1M DTT and 1µl (40units/µl) of RNAseOut (Invitrogen). This was mixed gently then incubated at room temperature for 2minutes. 1µl (200units) of SuperScript II Reverse Transcriptase was subsequently added and mixed by gentle pipetting. The mixture was then incubated further at room temperature for 10minutes followed by heating to 42°C for 50minutes. The reaction was finally inactivated by heating at 70°C for 15minutes.

In some experiments the cDNA was generated using the iScript cDNA generation kit (Biorad, Hemel Hempstead, UK). 4µl of 5x iScript select reaction mix were added to 2µl random primer, 1µg of RNA, 1µl iScript reverse transcriptase and nuclease free water to a total of 20µl. The mixture

was then incubated for 5 min at 25°C, 30minutes at 42°C then 5minutes at 85°C to inactivate the reverse transcriptase. The samples were stored at this stage between –20°C and +4°C.

2.1.7 Polymerase chain reaction (PCR) amplification of DNA

The Qiagen PCR Core Kit was used for the PCR. 2µl of cDNA was added to 10µM of each primer (MWG Biotech, Ebersberg, Germany), 2.5mM of dNTP, 2.5µl of 10x buffer, 5µl of Q buffer, 0.2µl of Taq polymerase and 10.375µl of water to a total volume of 25µl. The GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Warrington, Cheshire, UK) was used for amplification under specific conditions; 95°C for 5minutes, followed by 35 cycles of 95°C for 15seconds, 55-60°C for 30seconds then 72°C for 30seconds. This was followed by a final extension step at 72ºC for 10minutes, and then held at 4ºC.

2.1.8 Analysis of PCR products by agarose gel electrophoresis

The PCR products were analysed using a 2% agarose gel. This was prepared by mixing 2.4g of agarose powder and 120ml of 1x tris-borate-EDTA (TBE) (Sigma-Aldrich, Dorset, UK). The solution was heated in a microwave at full power for about three minutes to facilitate dissolution of the agarose. The difference in final volume was then corrected with distilled water. The solution was cooled under running tap water after which 5µl ethidium bromide (Sigma-Aldrich) was added to the solution. The ethidium bromide interacts with nucleic acids and fluoresces on exposure to ultraviolet light. The solution

was then poured in a plastic gel tray and allowed to set. The gel was then transferred to a horizontal gel electrophoresis system and immersed in 1x TBE buffer. 5µl of PCR product was prepared for loading by adding 1µl of DNA loading dye (Gelpilot Loading Dye 5x, Qiagen). φX174 DNA–Hae III Digest DNA ladder was also loaded onto the gel by addition to 3µl of a random PCR product (New England Biolabs, Hertfordshire, UK). Gel electrophoresis was carried out at 80V for 30minutes or until the PCR products can be visualised under an ultraviolet light source. The image was then captured using a gel documentation system (Syngene EV700, Cambridge, UK).

2.1.9 Purification of PCR product

Purification of PCR products was done using the Qiagen PCR purification kit. 100µl of binding buffer (PB Buffer) was added to each PCR product and the mixture was transferred into a spin column then centrifuged at 1200 rpm for two minutes. The effluent was discarded and 750µl wash buffer (PE Buffer) was added and centrifuged for a further two minutes. The effluent was discarded again and the column was centrifuged for another minute to ensure removal of any residual wash buffer. The purified DNA was then eluted in 20µl of distilled water and stored at -80°C.

2.1.10 Direct nucleotide sequencing

Bi-directional nucleotide sequencing was performed using the ABI PRISM® 310 Genetic Analyser (Applied Biosystems, Warrington, U.K.). A mixture was prepared for each sequencing reaction consisting of 2µl of purified

PCR product, 1µl of Big Dye (Applied Biosystems), 1µl of either forward or reverse primer, 1.5µl of 5X buffer and 4.5µl of nuclease free water (Sigma-Aldrich). The mixtures were placed in a 96-well plate and then incubated for amplification at 96°C for one minute followed by 25 cycles of incubation at 96°C for ten seconds, 50°C for ten seconds and 60°C for one minute, then held at 4°C. The amplified products were then purified using DNA precipitation. 25µl of 100% ethanol, 1µl of 125mM EDTA and 1µl of 3M sodium acetate were added carefully to the sequence mixture then incubated at room temperature for 15minutes followed by centrifugation at 2500rpm for thirty minutes at 4°C. The plate was then inverted to remove excess solution then centrifuged at 185rpm for 10seconds. 70µl of 70% ethanol was added, then centrifuged again at 1650rpm for 15minutes then inverted again with gentle centrifugation leaving behind the DNA pellet at the bottom of the wells. The plate was then left to dry at room temperature for 2minutes. 10µl of highly deiodinised (HiDi) formadide (Applied Biosystems) was then added to each well and dried on a heat block for 2minutes at 95°C. The plate was subsequently loaded into the ABI PRISM® 310 Genetic Analyser (Applied Biosystems) for sequencing analysis.

2.1.11 Quantitative real-time PCR using Taqman

Real-time q-PCR using Taqman was used to validate the microarray results by determining the expression of a gene of interest against the expression of one or more house-keeping genes. Taqman probes are fluorogenic probes with a reporter dye on the 5’ end and a quencher dye on the 3’ end. As Taq polymerase extends the forward primer, the probe is cleaved,

leading to the release of the reporter molecule from the close vicinity of the quencher. This results in an increase in fluorescence as PCR progresses. Taqman primers and probes (Applied Biosystems) were obtained for *FOS* Hs00170630_m1, *JUN* Hs01103582_s1, *IL1R2* Hs01030385_m1, *LAMC2* Hs01043707_m1, *GAL* Hs01032385_m1, *STAT1* Hs01014002_m1, *TNFSF13B* Hs00198106_m1, *IRF1* Hs00971960_m1, *VCAM1* Hs01003372_m1, *COL1A1* Hs01076756_g1, *COL4A1* Hs01007434_g1, *COL7A1* Hs01574745_g1, *COL17A1* Hs00166711_m1, *CCL18* Hs00268113_m1 and *HB-EGF* Hs00181813_ml purchased from Applied Biosystems (Foster City, CA, USA). *18S* was used as internal control (*18S* Hs03003631_g1) and water as a no template control to check for any contamination. The experiments were triplicated and the mean average for each sample was calculated.

For each 25µl reaction, 0.5µl of cDNA, 10.75µl of H_2O, 12.5µl of Taqman MasterMix (Applied Biosystems) and 1.25µl of Taqman assay was used making up a total volume of 25µl. The reaction mixes were carefully pipetted into a dedicated ninety-six well plate and heated to 50ºC, followed by 95ºC for 10minutes to activate AmpliTaq Gold, followed by 40 cycles of sample denaturation at 95ºC for 15seconds then annealing and extension at 60ºC for one minute using the ABI prism 7000 Sequence Detection System (Applied Biosystems). The gene expression was normalized against the expression of *18S* internal control and expressed relative to the average of control skin samples.

2.1.12 Whole genome gene expression microarray and data analysis

Gene expression microarray was performed using total RNA extracted from skin biopsy samples as outlined above. Whole genome gene expression microarray in each extracted RNA sample was performed using the Sentrix Human-6 Whole Genome Expression Beadchips (Illumina Inc, San Diego, CA, USA).

The data were normalised using a cubic spline function of Beadstudio software version 3.0 (Illumina Inc). Differential gene expression was calculated based on the expression difference score (DiffScore) of >13 or ≤13, which takes into account the background noise, sample variability and a differential fold change of two or greater. In addition, the average signal intensity for each probe was considered significant if its detection p value was <0.05. Any probe with a signal intensity p value of >0.05 was excluded from the analysis. Average signal intensity for each probe was calculated for both subjects (S) and controls (N). Fold change was calculated according to the formula S (average probe signal intensity) / N(average probe signal intensity).

2.1.13 Quantitative real-time PCR (qRT-PCR) using SYBR-Green

Quantitative real time polymerase chain reaction (qRT-PCR) using SYBR-Green q-PCR mastermix (Applied Biosystems) was performed to assess the expression of a genomic area of interest. Uracil-N-Glycosylase which is included in the SYBR-Green mastermix helps to remove any contaminating DNA that might still be present. SYBR-Green fluoresces when bound to double stranded DNA generated by the PCR and hence fluorescence intensity will be proportional to the PCR product concentration.

Two sets of collagen VII cDNA primers located at the 3' and 5' ends were designed to overlap exons in order to minimise the possibility of amplifying contaminating genomic DNA. Normal fibroblast cDNA was used as the comparative standard and water was used as a negative control sample to ensure there was no DNA contamination in the mixture. TaTa box binding protein (TBP) was used to as the normalisation control. The experiments were triplicated and the mean average calculated. Each 25µl reaction was composed of 12.5µl of 2x reaction buffer (contains dNTPS, HotGoldStar DNA polymerase, $MgCl_2$, Uracil-N-Glycosylase, SYBR Green I), 2.5µl (100nM) of forward and reverse primers, 6.5µl of water and 1µl (100ng) of cDNA template.

The reaction mixtures were carefully pipetted into dedicated PCR tubes and analysed using the Corbett Rotor-Gene 6000 series and the Corbett Rotor-Gene 6000 series software version 1.7. The optimum annealing temperature was initially determined by testing the primers using normal fibroblast cDNA, over a range of 58°C–68°C. The amplification conditions were two minutes at 50°C to activate uracil-N-glycosylase (UNG), followed by ten minutes at 95°C to inactivate UNG and activate HotGoldStar DNA polymerase. Subsequently, 45 cycles of fifteen seconds at 95°C (denaturation), thirty seconds at 60°C (annealing) and thirty seconds at 72°C (extension/DNA polymerisation phase) and a final extension at 72°C for ten minutes. The melting curve was read every 0.3°C between 72°C and 90°C.

2.2 Direct immunofluorescence microscopy studies

5μm frozen skin sections were cut from skin biopsies obtained from patients with EB. The skin sections were air-dried for 30minutes before being rehydrated in phosphate buffered saline (PBS) for a further 15minutes. The sections were then blocked with 20% goat serum (Sigma-Aldrich) for 20minutes and then incubated with mouse monoclonal anti- collagen VII antibody (clone LH 7.2; Sigma-Aldrich) diluted 1:1,000 in PBS, for 45minutes at 37ºC. The sections were then washed three times with PBS before being incubated with fluorescein isothyocyanate-labelled goat anti-mouse secondary antibody (Invitrogen). For a further 45minutes at 37ºC. Following PBS washes, the sections were air-dried and mounted with Vectashield Hard Set with Dapi (Vector Laboratories, Peterborough, UK) and viewed under a fluorescent microscope.

2.2.1 Quantification of collagen VII fluorescence

Mean fluorescence intensity was calculated for each sample using Image J (Rasband W.S., National Institutes of Health, Bethesda MD; http://rsb.info.nih.gov/ij/, 1997-2007) as described previously (Wong *et al.*, 2008). Ten measurements were taken at regular intervals using an area of 8x8 pixels and measuring every 50pixels along the DEJ, starting from the outermost edge of the image. The mean average values and standard errors were calculated for each image.

2.3 Transmission electron microscopy and morphometric analysis of anchoring fibrils at the DEJ

TEM was kindly performed by Mrs Patricia Dopping-Hepenstal at the Robin-Eady national epidermolysis bullosa diagnostic laboratory at St Thomas' Hospital. Skin biopsy specimens were cut and fixed in half-strength Karnovsky fixative for 4 hours at room temperature. After washing in 0.1M phosphate buffer, the samples were immersed in 1.3% osmium tetroxide (TAAB Laboratories, Berkshire, UK) for 2hours, followed by incubation in 2% uranyl acetate (BioRad) and gradually dehydrated using increasing concentrations of ethanol (50%, 70%, 95%) for 15minutes each time. Samples were then washed 3x 15minutes in 100% ethanol followed by 2x 15minutes in propylene oxide (TAAB laboratories). Similarly, increasing concentrations of resin/propylene oxide were used, 1:1 initially for 1hour then 3:1 overnight to gradually harden the samples. Following 2x 2hours of exposure to pure resin (812 embedding resin, medium, (TAAB laboratories) the samples were transferred into labelled moulds and allowed to polymerise for 18hours at 60°C. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100CX transmission electron microscope. Anchoring fibril numbers were determined over two separate 25-mm stretches of continuous lamina densa using the morphometric technique and anchoring fibril criteria previously described by Tidman and Eady (Tidman and Eady, 1985).

Special care was taken to dispose of radioactive uranyl acetate and osmium tetroxide into dedicated waste containers and also all equipment exposed to resin were polymerised before discarding as clinical waste. Following completion of handling radioactive material, the mini-Geiger meter

(Mini Instruments Ltd, Burnham on Crouch, Essex, UK) was used to monitor and record readings over dedicated areas in the laboratory for audit and health and safety purposes. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100CX transmission electron microscope.

2.4 Cell culture

2.4.1 HaCaT keratinocyte cell culture

HaCaT cells lines are spontaneously immortalized human keratinocytes (Boukamp *et al.*, 1988). The cells were cultured to 70–80% confluence in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum (FBS; Invitrogen).

2.4.2 Isolation of primary keratinocytes and fibroblasts from skin biopsies

Skin biopsies were immersed in EpiLife containing x1 concentration of streptomycin and penicillin antibiotics as well as amphotericin C (Invitrogen) for 1 hour at room temperature. Each sample was then cut into 2-3mm^2 pieces then incubated in 1mg/ml dispase overnight at 4°C (Roche Applied Science). To isolate keratinocytes the upper epidermal layer containing the keratinocytes was then separated from the dermis, vortexed at low speed for 1 minute and placed on mitomycin C-treated 3T3 cells. The flasks were then placed in a humidified cell culture incubator at 37°C in with 5% CO^2. EpiLife medium supplemented with defined growth supplement (EDGS; Invitrogen) was added and was refreshed every other day until the cell culture reached a near confluent state.

To isolate fibroblasts, the dermis was cut into very small pieces using sterile scalpel on a plastic tray and then immersed in 5ml trypsin-EDTA (Invitrogen). Tissue was then incubated at 37°C for 30 minutes and agitated every 10 minutes. Trypsin was inactivated by the addition of 10ml of 10% foetal bovine serum (FBS) (Biosera, Ringmer, East Sussex, UK). The suspension was then filtered through a cell strainer into a sterile falcon tube. The contents from the cell strainer contained the fibroblasts which were transferred carefully to a petri dish and incubated in collagenase D, 0.5mg/ml (Sigma-Aldrich) overnight at 37˚C in 5% CO^2 in air. The following day, the contents were filtered, centrifuged at 1000rpm for five minutes and the pellet re-suspended in fibroblast culture media consisting of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and 10% foetal calf serum (FCS, Biowest, East Sussex, UK). Passages two to five were used for the experiments.

2.4.3 Maintenance and passage of keratinocytes and fibroblasts

Primary fibroblast and keratinocyte cultures were maintained by changing the growth medium every other day. When culture cells reached near-confluence, they were washed with sterile PBS then passaged. This process includes the creation of a single cell suspension by incubating the cells in 5ml of trypsin-EDTA for 5minutes at 37˚C. An equivalent volume of DMEM containing 10% FCS was added to inactivate trypsin. The suspension was then centrifuged at 1000rpm for five minutes and the filtrate was aspirated carefully leaving the cell pellet at the bottom of the falcon tube. The cells were then

washed in sterile PBS then re-seeded into new flasks using a cell concentration ratio of 1:5 or alternatively stored in liquid nitrogen.

2.4.4 *In vitro* HB-EGF and TGFβ1 studies

Subconfluent cultures of keratinocytes and fibroblasts were fed with fresh DMEM medium without FBS supplementation and were cultured for 2hours before the addition of either recombinant human heparin binding epidermal growth factor (HB-EGF; Cat No.: 259-HE; R&D Systems; Abingdon, UK) or transforming growth factor beta 1 (TGFβ1; Cat No.: 240-B; R&D Systems). 10ng/ml of TGFβ1 and 100ng/ml of HB-EGF were added to the cells as described previously (Calonge *et al.*, 2004; Higashiyama *et al.*, 1993). Samples were then collected 15, 90 and 180minutes after the application of HB-EGF or TGFβ1.

2.5 Skin Biopsies

Informed written consent was obtained for 4mm punch biopsies. Using an aseptic technique, 1% lignocaine with adrenaline was injected into each of the biopsy sites. In most cases the biopsy specimen was immediately divided into three parts. One was placed into Michel's medium for future immunofluorescence studies, another into EM fixative and the third part into RNA later. Pressure was applied to the wound for haemostasis and suturing was not required. In view of skin fragility non-adhesive dressing was applied.

Chapter 3

Bullous dermolysis of the newborn: immunohistochemical, ultrastructural and molecular significance

Abstract

In patients with inherited skin blistering, the finding of intra-epidermal collagen VII immunostaining is often taken as a hallmark of a specific subtype of DEB; bullous dermolysis of the newborn (BDN), which usually has a good prognosis with spontaneous and sometimes complete clinical resolution. However, not all cases regress and there is a need for an improved clinicopathological and molecular correlation with better determination of prognosis. The aim of the study in this Chapter was to review cases of suspected EB referred to the National Diagnostic EB Laboratory over a 10-year period in which skin immunofluorescence microscopy revealed presence of intra-epidermal collagen VII labelling. The objective was to correlate the pattern of the collagen VII immunostaining with ultrastructural, molecular and clinical data and thereby improve understanding of the significance of intra-epidermal collagen VII labelling. Out of 570 skin samples reviewed, 35 cases (33 kindreds) were identified with intracytoplasmic collagen VII. BDN accounted for 14 cases while the remainder comprised other forms of dystrophic EB (n=10), junctional EB (n=4), EB simplex (n=4), aplasia cutis (n=1) and 2 undiagnosed cases. Variability in the pattern of intra-epidermal labelling as well as the ultrastructural morphology of intracytoplasmic inclusions was also noted in the different groups. In conclusion, intra-epidermal collagen VII immunolabelling in suspected cases of EB is not synonymous with a diagnosis of BDN. Careful assessment of the immunostaining pattern, in combination with ultrastructural, molecular and clinical data, is helpful in establishing more accurate diagnoses and prognoses.

3.1 Introduction

Bullous dermolysis of the newborn (BDN) is a rare variant of DEB first described in 1985 (Hashimoto *et al.*, 1985) in which there are well-defined immunohistochemical and ultrastructural abnormalities (Fine *et al.*, 2014). Direct immunofluorescence shows a variable reduction in collagen VII labelling at the DEJ as well as characteristic intracytoplasmic deposits of collagen VII within basal and suprabasal keratinocytes (Hashimoto *et al.*, 1985; Hashimoto *et al.*, 1989; Fine *et al.*, 1990). Ultrastructurally, there may be sub-lamina densa blistering with reduced or absent anchoring fibrils in combination with dilated rough endoplasmic reticulum (RER) and intracytoplasmic stellate bodies which represent perinuclear inclusions of collagen VII (Fine *et al.*, 1990; Hashimoto *et al.*, 1985; Hashimoto *et al.*, 1989). Proposed pathophysiological mechanisms in BDN include abnormal retention, secretion, transport or phagocytosis of collagen VII (Smith and Sybert, 1990; Fine *et al.*, 1993; Hammami-Hauasli *et al.*, 1998b; Fassihi *et al.*, 2006; Fassihi *et al.*, 2005).

The skin blistering in BDN, which usually presents at birth or in the neonatal period, typically improves markedly or even regresses completely within the first year of life. Thus, the condition was known initially as *transient* BDN (Hashimoto *et al.*, 1985). The clinical features, however, are not transient in all cases and therefore in the 2008 reclassification of EB, the word "transient" was dropped from the disease nomenclature (Fine *et al.*, 2008). Reports also pointed to variability in inheritance patterns with either autosomal dominant or recessive transmission (Christiano *et al.*, 1997b; Hammami-Hauasli *et al.*, 1998b; Fassihi *et al.*, 2005; Sawamura *et al.*, 2006a; Nakano *et*

al., 2007; Oh *et al.*, 2007; Almaani *et al.*, 2011; Almaani *et al.*, 2009; Hashikawa *et al.*, 2009; Murase *et al.*, 2011) (Table 3.1). Moreover, the immunohistochemical finding of intra-epidermal collagen VII labelling was found not to be exclusive to BDN (Table 3.2). It has also been reported as a transient finding during wound healing in recessive dystrophic EB (McGrath *et al.*, 1992), in the blister roof of some cases of dystrophic EB (Muramatsu *et al.*, 1999), in some forms of dystrophic EB other than BDN (Smith and Sybert, 1990; Phillips *et al.*, 1992; Konig *et al.*, 1994) and *in vitro* in association with certain dominant dystrophic EB mutations, for example p.Arg2008Gly (Chen *et al.*, 2002a) (Table 3.2).

Collectively, it is clear that BDN and intra-epidermal collagen VII do not represent a single clinicopathological and molecular entity. Therefore, in this Chapter, the implications of finding intra-epidermal collagen VII immunostaining in a diagnostic EB setting was studied with the aim of establishing a better understanding of the clinical and prognostic implications of this immunohistochemical finding.

Table 3.1 Reported *COL7A1* mutations associated with bullous dermolysis of the newborn. 9 dominant and 5 recessive *COL7A1* mutations have been described in BDN, the majority of which are missense glycine substitution mutations.

No.	Diagnosis	Allele 1				Allele 2				Reference
		exon/ intron	cDNA	protein	type	exon/ intron	cDNA	protein	type	
1	RDEB-BDN	Intron 5	682+1G>A	predicted retention of intron 5 + p.Pro228fsX32	SS	E68	c.5728G>A	p.Gly1910Ser	missense	(Hashikawa *et al.*, 2009)
2	DDEB-BDN	E18	c.2392G>A	p.Gly798Arg	MS	E75	c.6246del27	in frame deletion	SS	(Oh *et al.*, 2007)
3	DDEB-BDN	E35	c.4120-1G>C	predicted skipping of exon 35 (in-frame; 72aa)	SS	/	/	/	/	(Christiano *et al.*, 1997a)
4	DDEB-BDN	E42	c.4448G>A	p.Gly1483Asp	MS	/	/	/	/	(Almaani *et al.*, 2009)
5	RDEB-BDN	E44	c.4556G>A	p.Gly1519Asp	MS	E86	c.6752G>A	p.Gly2251Glu	missense	(Hammami-Hauasli *et al.*, 1998a)
6	DDEB-BDN	E45	c.4565G>A	p.Gly1522Glu	MS	/	/	/	/	(Fassihi *et al.*, 2005)
7	DDEB-BDN	E54	c.5017G>A	p.Gly1673Arg	MS	/	/	/	/	(Frew *et al.*, 2011)

8	RDEB-BDN	E73	c.6023G>A	p.Arg2008His	MS	E64	c.5504delA c.2969G>A	p.Asp1835fsX14 p.Arg990Gln	PTC	(Nakano *et al.*, 2007)
9	DDEB-BDN	E73	c. 6110G>A	p.Gly2037Glu	MS	/	/	/	/	(Sawamura *et al.*, 2006b)
10	DDEB-BDN	E74	c.6189-6206del18	p.Gly2064-Arg2069del	in frame deletion	/	/	/	/	(Wertheim-Tysarowska *et al.*, 2012)
11	DDEB-BDN	E81	c.6557G>A	p.Gly2186Glu	MS	/	/	/	/	(Almaani *et al.*, 2011)
12	DDEB-BDN	E85	c. 6725G>A	p.Gly2242Glu	MS	/	/	/	/	(Murase *et al.*, 2011)
13	RDEB-BDN	E93	c.7115G>T	p.Gly2372Val	MS	E93	c.7115G>T	p.Gly2372Val	missense	(Almaani *et al.*, 2011)
14	RDEB-BDN	E112	c.8341G>A	p.Gly2781Arg	MS	E51	c.4894C>T	p.Arg1632Stop	PTC	(Almaani *et al.*, 2011)

BDN- bullous dermolysis of the newborn, DDEB- dominant dystrophic epidermolysis bullosa; RDEB- recessive DEB; MS-missense; SS-splice site; PTC-premature termination codon.

Table 3.2 Reported cases of BDN and dystrophic EB associated with intracytoplasmic retention of collagen VII including BDN. The literature contains 19 publications (33 subjects) describing BDN with intracytoplasmic retention of collagen VII, as well as 11 publications (20 subjects) of non-BDN dystrophic EB cases.

No.	Diagnosis	Mode of inheri-tance	Mutations	Collagen VII Immunolabelling	Electron Microscopy	Clinical Outcome	Reference
1	BDN	?AD	ND	prominent focal granular intracytoplasmic retention of collagen VII and interrupted collagen VII labelling along the DEJ	ND	complete resolution	(Radkevich-Brown and Shwayder, 2013)
2	BDN	?AD	ND	focal granular intracytoplasmic retention of collagen VII	ND	complete resolution	(Radkevich-Brown and Shwayder, 2013)
3	BDN	sporadic	ND	no intracytoplasmic retention of collagen VII	ND	complete resolution	(Radkevich-Brown and Shwayder, 2013)
4	BDN	?AR	ND	prominent focal granular intracytoplasmic retention of collagen VII	ND	complete resolution, nail dystrophy	(Radkevich-Brown and Shwayder, 2013)
5	DEB	ND	ND	absent collagen VII labelling at DEJ with intracytoplasmic retention of collagen VII	ND	ND	(Berk *et al.*, 2013)

6	RDEB-I	AR	p.Gly2695Ser/ p.Gly2695Ser	intracytoplasmic retention of collagen VII in basal keratinocytes	reduced numbers of abnormal or rudimentary AF. RER vesicles were seen	chronic involvement of the inversa pattern	(van den Akker *et al.*, 2011)
7	RDEB-I	AR	p.Gly2602Glu/ p.Gly1907Glu	intracytoplasmic retention of collagen VII in basal keratinocytes	reduced numbers of abnormal or rudimentary AF	chronic involvement of the inversa pattern	(van den Akker *et al.*, 2011)
8	RDEB-I	AR	p.Gly1761Ala/ p.Gly1761Ala	intracytoplasmic retention of collagen VII in basal keratinocytes	reduced numbers of abnormal or rudimentary AF	improved initially within a few months of life then worsened by 1 year	(van den Akker *et al.*, 2011)
9	BDN	ND	ND	granular deposits within the epidermis	ND	reduced blistering	(Oppenheimer and Hallas, 2011)
10	BDN	AD	p.Gly1673Arg	granular deposits within the epidermis	reduced AF with dilated RER and stellate bodies in basal keratinocytes	complete resolution	(Frew *et al.*, 2011)
11	BDN	AD	p.Gly2242Glu	granular deposits within the epidermis	reduced AF with stellate bodies in basal and suprabasal keratinocytes	complete resolution	(Murase *et al.*, 2011)
12	BDN	AR	c.682+1G>A/ p.Gly1910Ser	granular deposits within basal keratinocytes	reduced and poorly formed AF. Large stellate bodies in basal keratinocytes	complete resolution	(Hashikawa *et al.*, 2009)
13	BDN	AR	p.Gly798Arg/ c.6246del27	ND	ND	complete resolution	(Oh *et al.*, 2007)

14	BDN	AR	c.5504delA/ p.Arg2008His	ND	reduced and poorly formed AF. Dilated RER in the basal and suprabasal layers	complete resolution	(Nakano *et al.*, 2007)
15	BDN	AD	p.Gly2037Glu	intracytoplasmic deposits within the epidermis	reduced AF	reduced blistering	(Sawamura *et al.*, 2006b)
16	BDN	AD	p.Gly1522Glu	widespread and granular deposits within the epidermis	reduced and poorly formed AF. Large, granular perinuclear stellate bodies in basal and suprabasal keratinocytes	complete resolution	(Fassihi *et al.*, 2005)
17	BDN	AD	p.Gly1522Glu	focal intraepidermal deposits of collagen VII within some basal keratinocytes	AF reduced, thin and wispy. Small intracytoplasmic inclusions in some basal keratinocytes.	complete resolution	(Fassihi *et al.*, 2005)
18	BDN	AD	p.Gly1522Glu	focal intraepidermal deposits of collagen VII within some basal keratinocytes	AF reduced, thin and wispy. Small intracytoplasmic inclusions in some basal keratinocytes	complete resolution	(Fassihi *et al.*, 2005)
19	RDEB-O	AD	p.Gly2316Arg/ p.Gly2287Arg	weak labelling at BM with basal and suprabasal retention of collagen VII at 3 days of age. At 10 years; normal BM labelling with infrequent punctuate labelling within the epidermis	ND	reduced blistering	(Shimizu *et al.*, 1999)

20	BDN	AR	p.Gly1519Asp / p.Gly2551Glu	basal and suprabasal retention of collagen VII	Reduced and abnormal AF. Dilated RER in keratinocytes with vacuoles containing electron dense and filamentous aggregates.	reduced blistering	(Hammami-Hauasli *et al.*, 1998a)
21	BDN	AD	c.4120-1G>C	granular intracytoplasmic staining in basal keratinocytes	ND	complete resolution	(Christiano *et al.*, 1997a)
22	BDN	sporadic	ND	intense intracytoplasmic collagen VII	sublamina densa blister	complete resolution	(Hanson *et al.*, 1999)
23	BDN	sporadic	ND	granular deposits within the epidermis	subepidermal vesicles and decreased numbers of AF	reduced blistering	(Hanson *et al.*, 1999)
24	BDN	sporadic	ND	focal granular deposits within the epidermis	subepidermal vesicles and decreased numbers of AF	complete resolution	(Hanson *et al.*, 1999)
25	BDN	sporadic	ND	abundant but patchy intraepidermal collagen staining	subepidermal blister, reduced rudimentary AF. Basal and suprabasal keratinocytes contain granular perinuclear inclusion bodies	reduced blistering	(Hatta *et al.*, 1995)
26	DDEB-gen	AD	ND	intracytoplasmic expression of collagen VII in cultured keratinocytes stimulated by TGF-β2	dilated RER cisternae of vesicular appearance filled with electron dense content	reduced blistering	(Konig *et al.*, 1994)
27	RDEB-I	sporadic	ND	ND	AFs show variable degrees of abnormality	continued blistering	(Gedde-Dahl and Winberg, 1994)

28	BDN	sporadic	ND	granular intracytoplasmic deposits	intracytoplasmic retention of collagen VII	complete resolution	(Fine *et al.*, 1993)
29	BDN	ND	ND	granular intracytoplasmic deposits	intracytoplasmic retention of collagen VII	complete resolution	(Fine *et al.*, 1993)
30	BDN	ND	ND	ND	ND	complete resolution	(Fine *et al.*, 1993)
31	BDN	ND	ND	granular intracytoplasmic deposits	ND	reduced blistering	(Fine *et al.*, 1993)
32	BDN	ND	ND	ND	ND	complete resolution	(Fine *et al.*, 1993)
33	BDN	AD	ND	granular intracytoplasmic deposits	ND	reduced blistering	(Fine *et al.*, 1993)
34	BDN	AD	ND	ND	ND	NA	(Fine *et al.*, 1993)
35	BDN	AD	ND	ND	ND	reduced blistering	(Fine *et al.*, 1993)
36	BDN	AD	ND	ND	ND	reduced blistering	(Fine *et al.*, 1993)

37	BDN	sporadic	ND	granular deposits in all layers of the epidermis	abnormal AF. Dilated RER, containing amorphous material and electron dense stellate bodies	complete resolution	(Okuda *et al.*, 1993)
38	BDN	sporadic	ND	intracytoplasmic deposits within keratinocytes	reduced AF. Deposits of a granular material with focal densities seen within the RER of the keratinocytes	reduced blistering	(Phillips *et al.*, 1992)
39	DEB	sporadic	ND	patchy intracytoplasmic deposits within keratinocytes	AF sparse and wispy. No split or granular deposits were seen	blisters continues at 2m, milia scarring, nails unaffected	(Phillips *et al.*, 1992)
40	DDEB-pt	AD	ND	granular intracytoplasmic deposits within basal keratinocytes	normal AF. Lipid droplets were present in basal keratinocytes but no granular deposits were seen	reduced blistering	(Phillips *et al.*, 1992)
41	DEB	?AD	ND	prominent intraepidermal deposits	reduced AF. Dilated RER containing granular deposits within keratinocytes.	reduced blistering	(Phillips *et al.*, 1992)
42	DEB	?AD	ND	few discrete deposits within the epidermis	abnormal AFs. No intracytoplasmic granular deposits could be found	pregnancy terminated	(Phillips *et al.*, 1992)

43	RDEB-sev gen	AR	ND	absent collagen VII staining pre-wounding. At day 10-13 intracytoplasmic retention of collagen VII was noted in basal and suprabasal keratinocytes.	ND	ND	(McGrath *et al.*, 1992)
44	BDN	sporadic	ND	ND	reduced and abnormal AF. Dilated RER containing filamentous aggregates. cell membrane-bound structures with phagocytosed collagen VII was noted	complete resolution	(Eng *et al.*, 1991; Hashimoto and Eng, 1992)
45	DDEB	AD	ND	granular perinuclear intracytoplasmic deposits	sublamina densa blister	reduced blistering	(McCollough *et al.*, 1991)
46	RDEB	AR	ND	intracytoplasmic retention of collagen VII in basal and suprabasal keratinocytes	abnormal AFs, dilated RER cisternae with vesicles filled with electron dense content	ND	(Smith and Sybert, 1990)
47	DEB	ND	ND	granular intracytoplasmic deposits within basal keratinocytes	markedly reduced AF. Amorphous perinuclear basilar cytoplasmic deposits.	only one blister since aged 6 months; no further skin fragility.	(Fine *et al.*, 1990)
48	DDEB	AD	ND	granular intracytoplasmic deposits within basal keratinocytes	ND	complete resolution	(Fine *et al.*, 1990)
49	DDEB	AD	ND	granular intracytoplasmic deposits within basal keratinocytes	ND	complete resolution	(Fine *et al.*, 1990)

50	DEB	ND	ND	granular intracytoplasmic deposits within basal keratinocytes	ND	complete resolution	(Fine *et al.*, 1990)
51	DEB	sporadic	ND	ND	reduced AF. stellate inclusions within basal and suprabasal keratinocytes	complete resolution	(Hashimoto *et al.*, 1989)
52	DEB	ND	ND	ND	reduced AF. stellate inclusions within basal and suprabasal keratinocytes	complete resolution	(Hashimoto *et al.*, 1989)
53	BDN	sporadic	ND	ND	reduced AF. Dilated RER. Vacuoles containing electron dense amorphous material within basal keratinocytes	complete resolution	(Hashimoto *et al.*, 1985)

DEB, dystrophic epidermolysis bullosa, DDEB, dominant DEB; DDEB-pt, DDEB-pretibial; BDN, bullous dermolysis of the newborn; RDEB, recessive DEB; RDEB-I, RDEB-inversa, RDEB-O, RDEB-other; RDEB-sev gen, RDEB severe generalised; AD, autosomal dominant; AR, autosomal recessive; ND, not determined; AF, anchoring fibrils; RER, rough endoplasmic reticulum; BM, basement membrane.
Note: the following mutations were found to result in intracytoplasmic retention of collagen VII in in vitro studies: p.Arg2008Gly (Hammami-Hauasli et al. J Biol Chem 1998; 273: 1928-1934, Chen et al. J Biol Chem 2002; 277: 2118-2124, Woodley et al. J Biol Chem 2008; 283: 17838-17845), p.Gly1776Arg (Fritsch et al. Biol Chem 2009; 284: 30248-30256), p.Gly2006Asp & p.Gly2015Glu (Bruckner-Tuderman et al. Matrix Biol. 1999; 18:43-54, Fritsch et al. Biol Chem 2009; 284: 30248-30256), p.Gly2034Arg (Bruckner-Tuderman et al. Matrix Biol. 1999;18:43-54).

3.2 Patient selection and methods

I reviewed the reports of 570 skin samples sent to the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory in London over a 10 year-period for the investigation of a possible subtype of EB. The population investigated included white Caucasians, Middle-Eastern, South-East Asian and Asian subjects. Immunofluorescence (IF) microscopy reports were examined from all cases demonstrating intra-epidermal collagen VII on IF microscopy. The ultrastructural findings were re-examined in those cases to review the anchoring fibrils and intracytoplasmic inclusions. The clinical information was obtained from the clinical notes where possible, details supplied with the original referral letters or the EB diagnostic lab logbook. Detailed follow up information was not available for some patients, particularly in historic cases and ones from overseas. Immunofluorescence studies, transmission electron microscopy and DNA sequencing analysis had been performed previously as described in Chapter 2.

3.3 Results

570 archival EB skin biopsy reports were reviewed and intracytoplasmic retention of collagen VII on immunofluorescence microscopy was documented in 35 cases (33 kindreds).

Overall, several different patterns of collagen VII labelling were identified within the epidermis and along the basement membrane. The distribution of intra-epidermal collagen VII labelling ranged from basal only, basal and suprabasal to pan-epidermal and in the majority of cases the more focal intra-epidermal labelling was concentrated above the dermal papillae.

Furthermore, most cases demonstrated reduced levels of collagen VII expression along the DEJ, although labelling ranged from normal to completely absent.

The first question was to ask what type(s) of EB the intra-epidermal collagen VII occurred in. Using a combination of IF microscopy, TEM, the clinical features and *COL7A1* (and other) gene analysis, I was able to sub-divide the 35 cases into 14 cases of BDN, 1 case of EB pruriginosa (EB-pr), 9 cases of recessive dystrophic EB (RDEB), 4 cases of EB simplex (EBS), 4 cases of junctional EB (JEB), 1 case of aplasia cutis congenita and 2 undiagnosed cases. Full details are shown in Tables 3.3 and 3.4. Of note, these findings indicated that intra-epidermal collagen VII was not exclusive to BDN or even other variants of dystrophic EB.

I then assessed whether the intra-epidermal collagen VII appearances by IF microscopy alone represented a useful means of sub-classifying the diagnosis and prognosis (Figure 3.2). Of the 14 cases diagnosed as BDN, the intra-epidermal collagen VII labelling along the basal layer was focal in 13 cases; in only 1 case was there a more diffuse staining appearance. In 10 cases the intra-epidermal labelling occurred in basal and suprabasal keratinocytes, whereas in the other 3 it was pan-epidermal (Figure 3.3). Clinically, 11 out of 14 cases of BDN showed complete resolution of skin fragility as indicated by the clinician's supplied information, while blistering improved but did not resolve completely in 3 cases (follow up information was available beyond 1 year for 2 patients but only for 4 months in the 3rd case). With regards to prognosis in BDN, however, the IF microscopy appearances *per se* did not correlate with the final clinical outcome.

Next I examined whether any of the other non-BDN cases had intra-epidermal collagen VII labelling patterns on IF that overlapped with the cases of BDN or if a complete distinction was possible (Figures 3.4 and 3.5). Based on the expression of collagen VII along the DEJ, non-EB cases had completely normal collagen VII immunostaining, whereas non-BDN cases of DEB showed variable levels of expression. DEB cases associated with absent collagen VII immunostaining or a moderate reduction of labelling in association with a loss of function mutation on both alleles developed RDEB-sev gen. on the other hand, cases with mild to moderate reduction generally developed BDN or RDEB-O depending on the degree of intra-epidermal expression of collagen VII. Generally, pan-epidermal retention of collagen VII was associated with a milder phenotype. This might indicate that the extent of intra-epidermal retention of collagen VII in BDN cases is sufficient to restore collagen VII expression as it is eventually secreted into the DEJ, whereas the level of retention is insufficient to normalize the severely reduced collagen VII expression in non-BDN cases. This would support observations that defective storage and delayed secretion/transport of collagen VII underlie the pathology in BDN rather than phagocytosis of collagen VII (Smith and Sybert, 1990; Fine *et al.*, 1993; Hammami-Hauasli *et al.*, 1998b; Fassihi *et al.*, 2006; Fassihi *et al.*, 2005).

Immunohistochemical and ultrastructural features typically associated with BDN were noted (Figure 3.1), including intracytoplasmic retention of collagen VII within the basal and suprabasal layers of the epidermis on immunofluorescence, as well as, dilated RER and peri-nuclear granular stellate bodies on electron microscopy. Although immunohistochemical variations

were noted (Figure 3.3) typical stellate bodies were identified in 7 out of 14 cases of BDN (TEM was not performed in 4 cases). There were no clear immunohistochemical or ultrastructural differences in the cases of BDN that failed to have complete clinical resolution compared to those that resolved completely.

On the other hand intracytoplasmic retention of collagen VII was associated with atypical inclusion bodies in other forms of DEB and non-DEB cases as well as normal skin, ranging from empty vacuoles to homogenous or granular inclusion bodies.

Figure 3.1 Classical immunohistochemical and ultrastructural features of BDN in a patient heterozygous for the *COL7A1* splice site deletion mutation IVS73-3del6. (A) IF studies show collagen VII labelling at the DEJ (arrows) as well as intracytoplasmic retention of collagen VII within the basal and suprabasal layers of the epidermis. (B) TEM reveals dilated RER, and large peri-nuclear stellate bodies (asterix) containing granular inclusions. There is also a reduced number of anchoring fibrils beneath the lamina densa. Bars in a= 50 μm; bars in b= 2.5 μm.

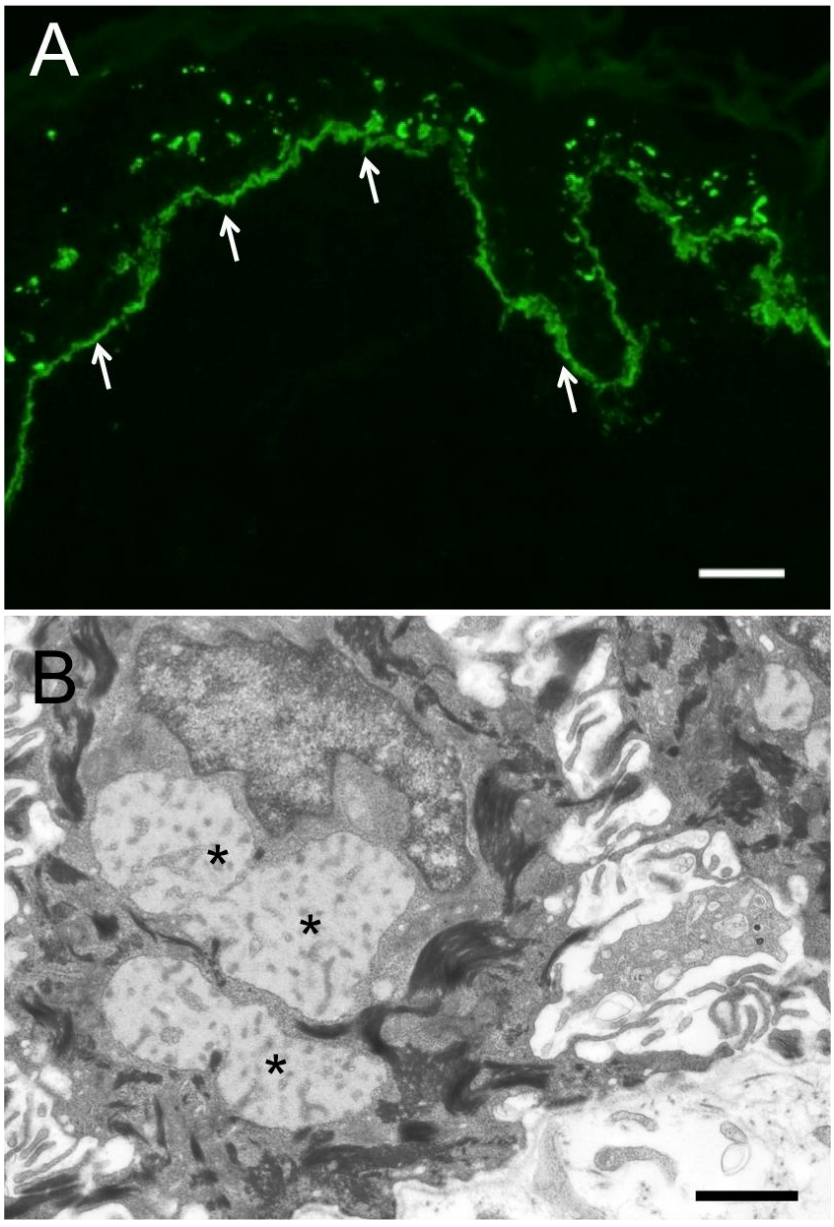


Table 3.3 Molecular pathology, immunofluorescence and ultrastructural findings associated with cases of bullous dermolysis of the newborn identified in this study.

No.	Diagnosis	Molecular Pathology				Mode of inherit-ance	Age at biopsy	Clinical features	Pattern of Collagen VII Staining		Ultrastructural findings on electron microscopy	
		gene	mutation(s)	exon/ intron	type				DEJ labelling pattern	epidermal labelling pattern	anchoring fibrils	intracellular inclusions
1	DDEB-BDN	*COL7A1*	p.Gly1522Glu/-[a]	E45	MS	AD	2m	blistering ceased at 4m with residual milia	reduced, maps to roof of blister	focal pan-epidermal	reduced, wispy	typical granular stellate bodies in basal, suprabasal and spinous layers
2	RDEB-BDN	*COL7A1*	c.2021insG/ ND	E73	PTC/ ND	ND	7d	blistering reduced but was still present at 1 year	reduced, maps to roof of blister	focal pan-epidermal	reduced, wispy	numerous typical granular stellate bodies in basal, suprabasal, spinous layers
3	RDEB-BDN	*COL7A1*	p.Arg2008His/ p.Arg2008His	E73	MS/ MS	AR	3d	blistering ceased at 7m	reduced, maps to roof of blister	focal basal/ suprabasal	reduced, wispy	atypical homogenous perinuclear inclusion bodies
4	DDEB-BDN	*COL7A1*	IVS73-3del6/-	I73	SS	AD*	17d	blistering ceased at 5m with residual scarring and milia	reduced, no blister	focal basal/ suprabasal	reduced, wispy	numerous typical granular stellate bodies

5	DDEB-BDN	*COL7A1*	p.Gln2072Arg/-	E74	MS	AD	14d	blistering improved but did not resolve completely at 15 months	reduced, maps to roof of blister	focal basal/ suprabasal	ND	ND
6	DDEB-BDN	*COL7A1*	p.Gly2186Glu/-[b]	E81	MS	AD*	2m	blistering ceased at 2m	reduced, no blister	focal basal/ suprabasal	reduced, wispy	dilated RER and typical granular stellate bodies in basal, suprabasal layers
7	DDEB-BDN	*COL7A1*	p.Arg2287Arg/-	E87	MS	AD*	18d	complete resolution of blisters without milia or nail changes	reduced, maps to roof of blister	focal basal/ suprabasal	reduced, wispy	single typical granular stellate body
8	RDEB-BDN	*COL7A1*	p.Gly2372Val/ p.Gly2372Val[b]	E93	MS/ MS	AR	5 days	blistering ceased at 3 weeks	reduced, no blister	generalised basal/ suprabasal	reduced, wispy	dilated RER and typical perinuclear granular stellate bodies
9	RDEB-BDN	*COL7A1*	p.Gly2372Val/ p.Gly2372Val[b]	E93	MS/ MS	AR	7 days	complete resolution	reduced maps to roof of blister	focal basal/ suprabasal	ND	ND
10	RDEB-BDN	*COL7A1*	p.Gly2781Ser/ p.Arg1632Stop[b]	E112/ E51	MS/ PTC	AR	1 month	blistering reduced but was still present at 4m	reduced, no blister	focal basal/ suprabasal	reduced, wispy	single typical perinuclear granular stellate body

11	BDN	ND	ND	ND	ND	ND	9 days	complete resolution	reduced, maps to roof of blister	focal pan-epidermal	reduced, wispy	typical granular stellate bodies
12	BDN	ND	ND	ND	ND	ND	1m	complete resolution	reduced, no blister	focal basal/ suprabasal	reduced, wispy	ND

*de novo; ND -not determined; EB, epidermolysis bullosa; DEB, dystrophic EB; DDEB, dominant DEB; BDN, bullous dermolysis of the newborn; RDEB, recessive DEB; DEJ, dermal-epidermal junction; IVS, intervening sequence (intron); MS, missense; PTC, premature termination codon; SS, splice site; AD, autosomal dominant; AR, autosomal recessive. [a] Three members of the same family (3 generations) Fassihi *et al*. *Br J Dermatol.* 2005; 153:1058-63; [b] Almaani *et al*. Acta Derm Venereol. 2011;91:262-6.

Figure 3.2 A schematic outlining a possible correlation between the patterns of immunofluorescence labelling and subsequent diagnosis in cases of intracytoplasmic retention of collagen VII.

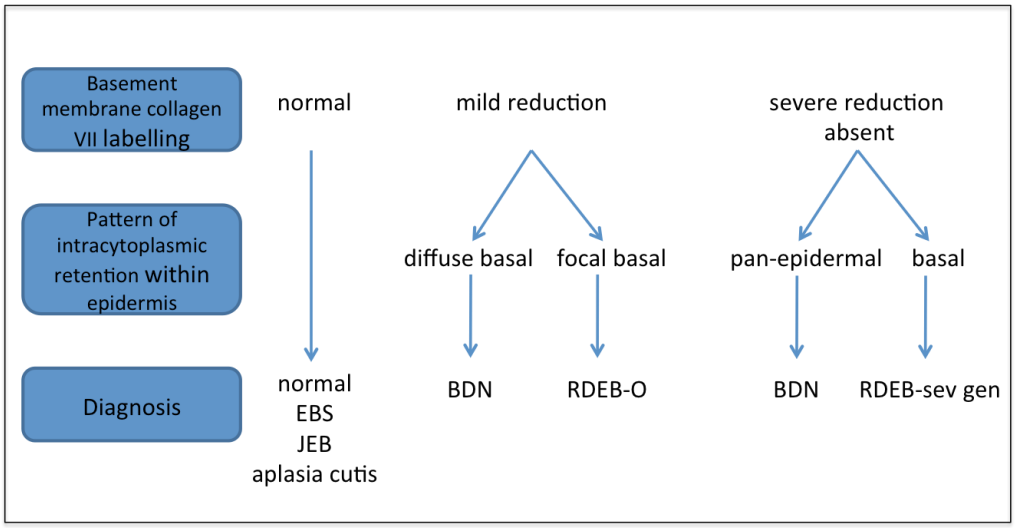
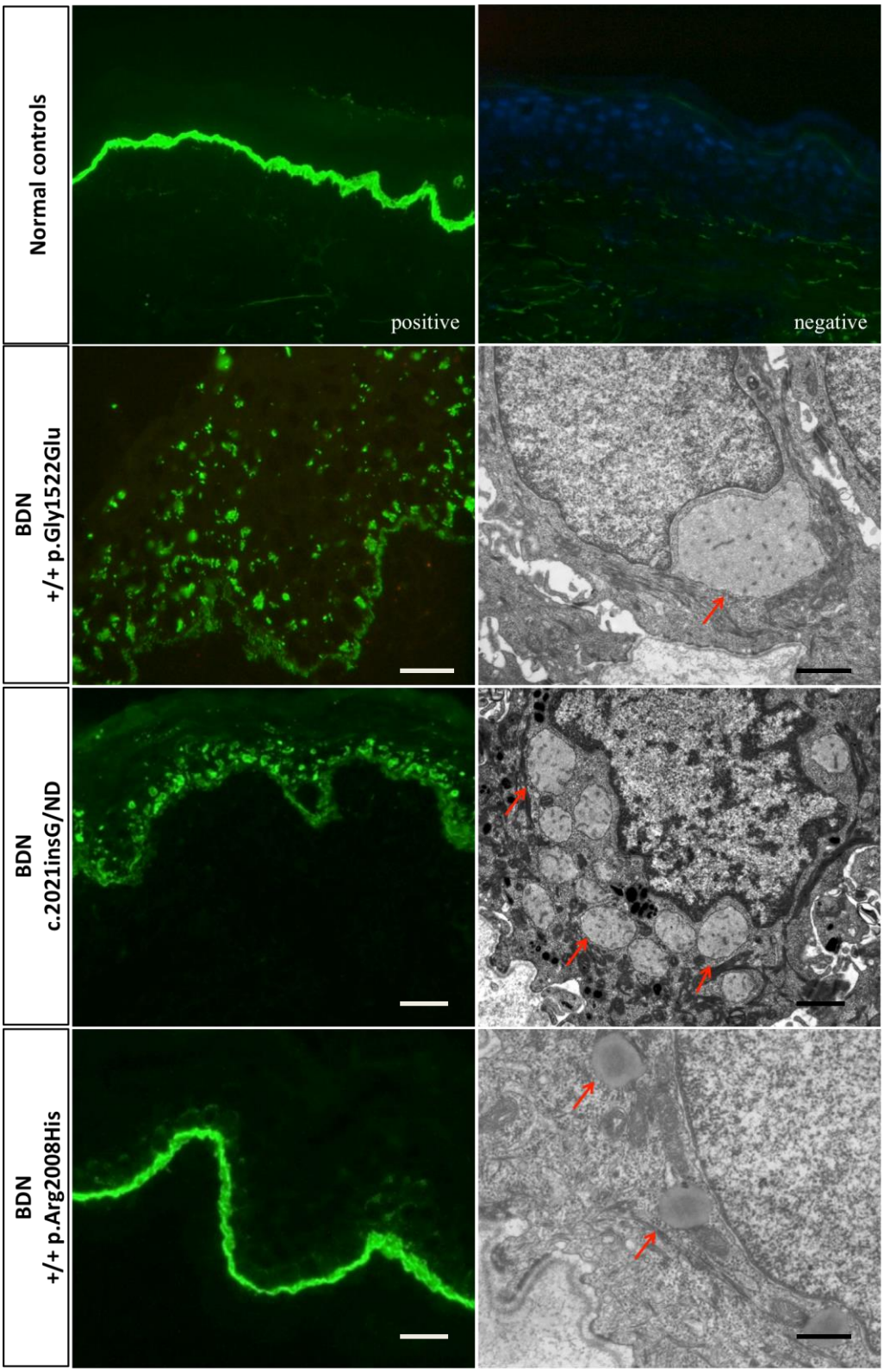
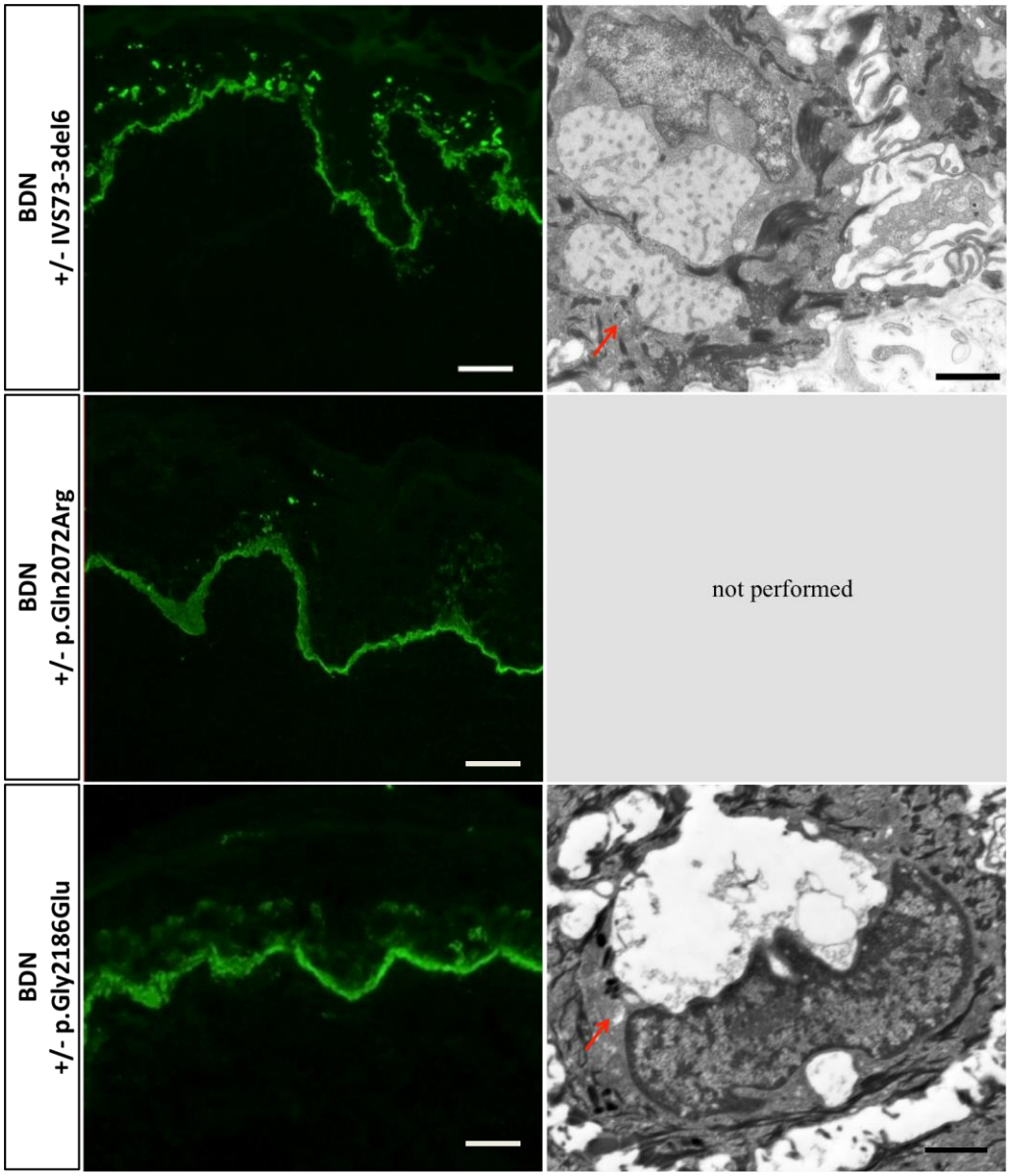
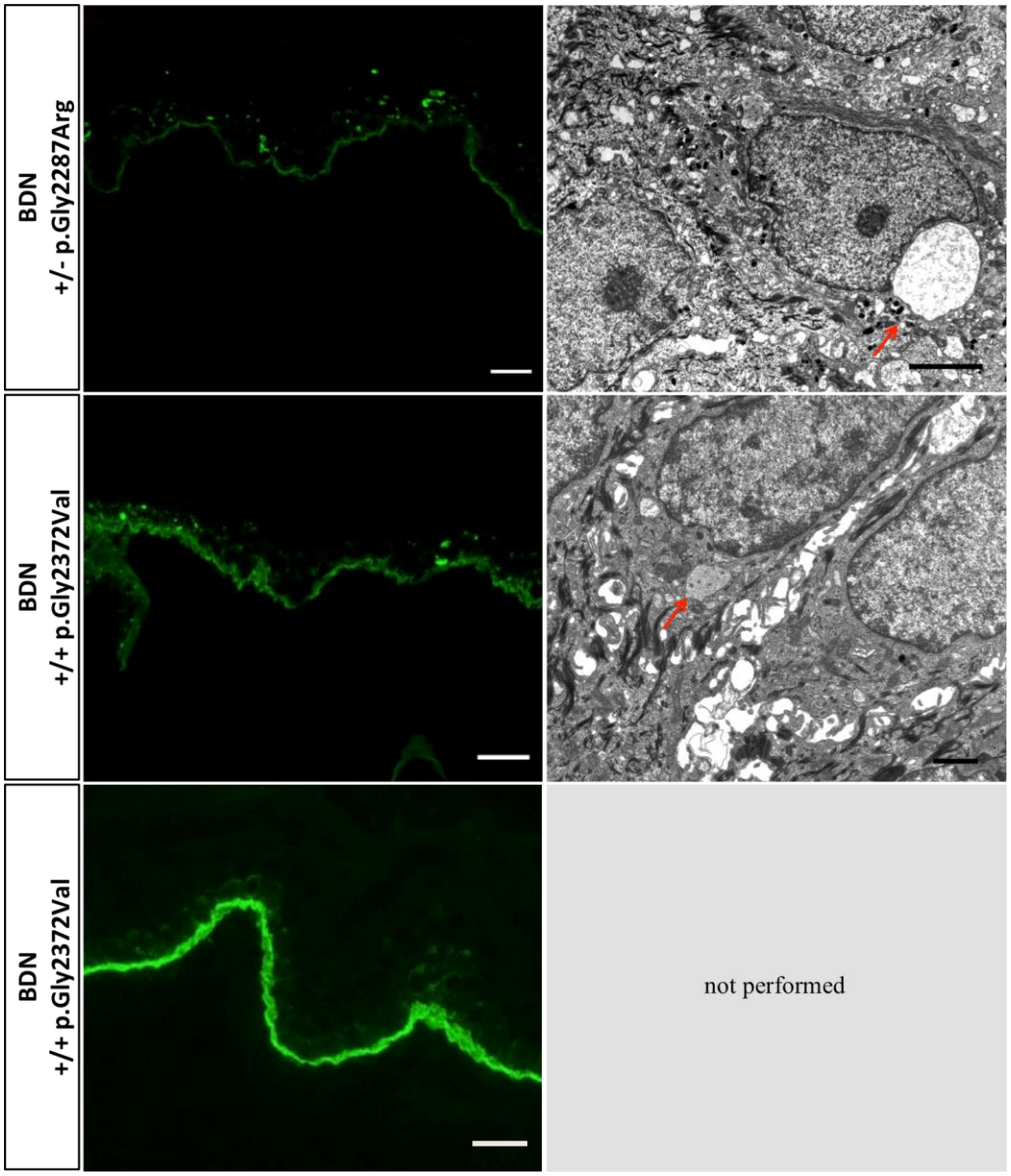


Figure 3.3 immunohistochemical and ultrastructural appearances of BDN cases analysed in this study. Descriptive details of the DIF and TEM findings are presented in Table 3.3. IMF bars = 50 μm; TEM bars = 2.5 μm.




BDN
+/- IVS73-3del6
BDN
+/- p.Gln2072Arg
not performed
BDN
+/- p.Gly2186Glu


BDN
+/- p.Gly2287Arg
BDN
+/+ p.Gly2372Val
BDN
+/+ p.Gly2372Val
not performed

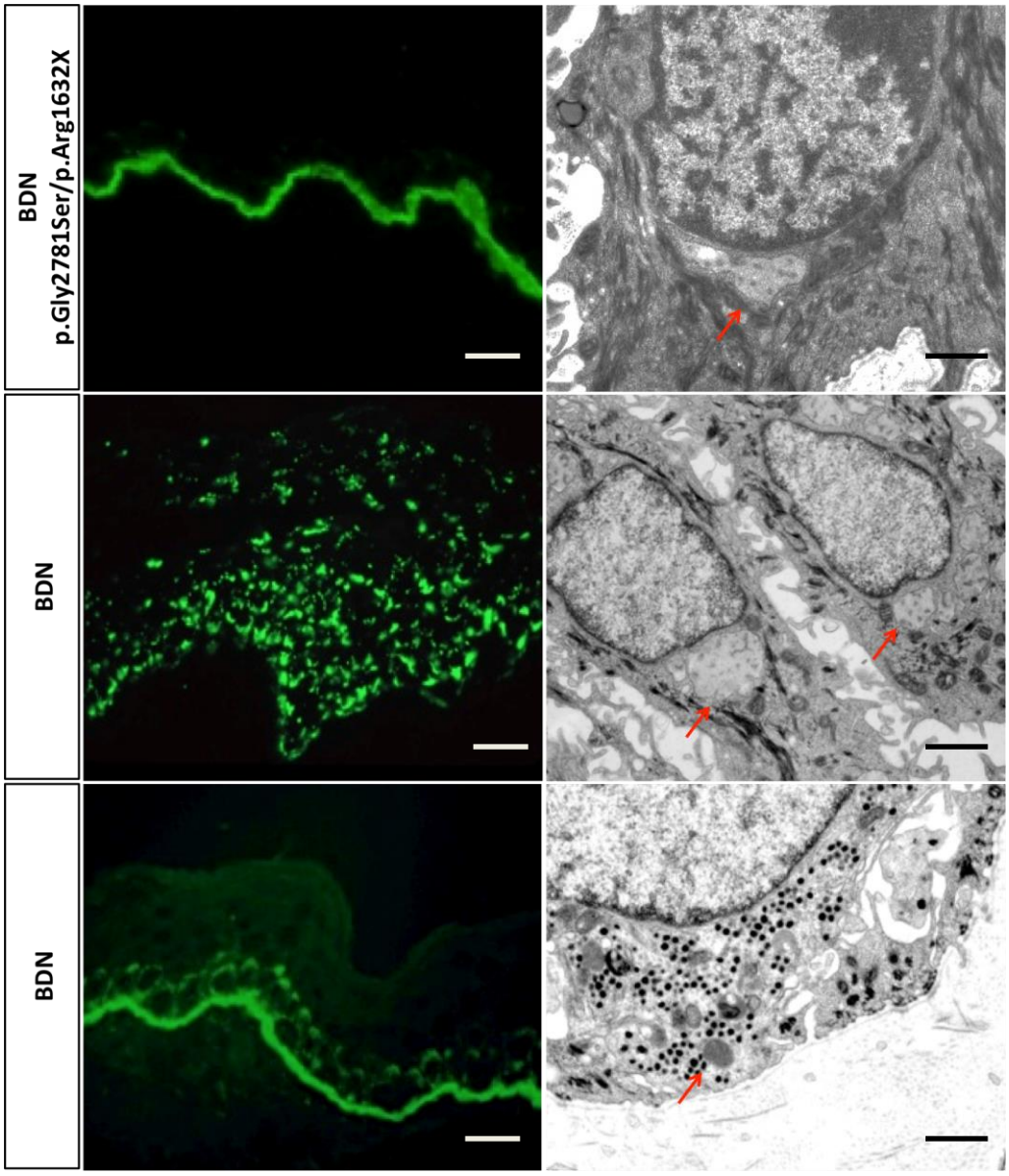

BDN
p.Gly2781Ser/p.Arg1632X
BDN
BDN

Table 3.4 Non-BDN cases associated with intracytoplasmic retention of collagen VII identified in this study.

No.	Diagnosis	Molecular Pathology				Mode of inherit-ance	Complete resolution	Pattern of Collagen VII Staining		Ultrastructural findings on electron microscopy	
		gene	mutation(s)	exon/ intron	type			DEJ labelling pattern	epidermal labelling pattern	anchoring fibrils	intracellular inclusions
Dystrophic epidermolysis bullosa											
1	Dominant EB-pr	ND	ND	ND	ND	ND	No	normal	generalised basal/ suprabasal	ND	ND
2	RDEB-O	*COL7A1*	p.Gly1347Trp/ p.Gly1347Trp	E34	MS/MS	AR	No	reduced, maps to roof of blister	focal basal/ suprabasal	ND	ND
3	RDEB-O	*COL7A1*	p.Gly1347Trp/ p.Gly1347Trp	E34	MS/MS	AR	No	reduced, maps to roof of blister	focal basal/ suprabasal	ND	ND
4	RDEB-O	*COL7A1*	p.Gly1483Asp/ p.Gly1483Asp[a]	E42	MS/MS	AR	No	reduced, no blister	focal basal/ suprabasal	reduced, wispy	atypical homogenous inclusion bodies
5	RDEB-O	*COL7A1*	**IVS55-1G>T/ IVS55-1G>T**	I55	SS/SS	AR	No	reduced, maps to roof of blister	focal basal/ suprabasal	reduced, wispy	atypical homogenous inclusion bodies in basal and suprabasal layers.

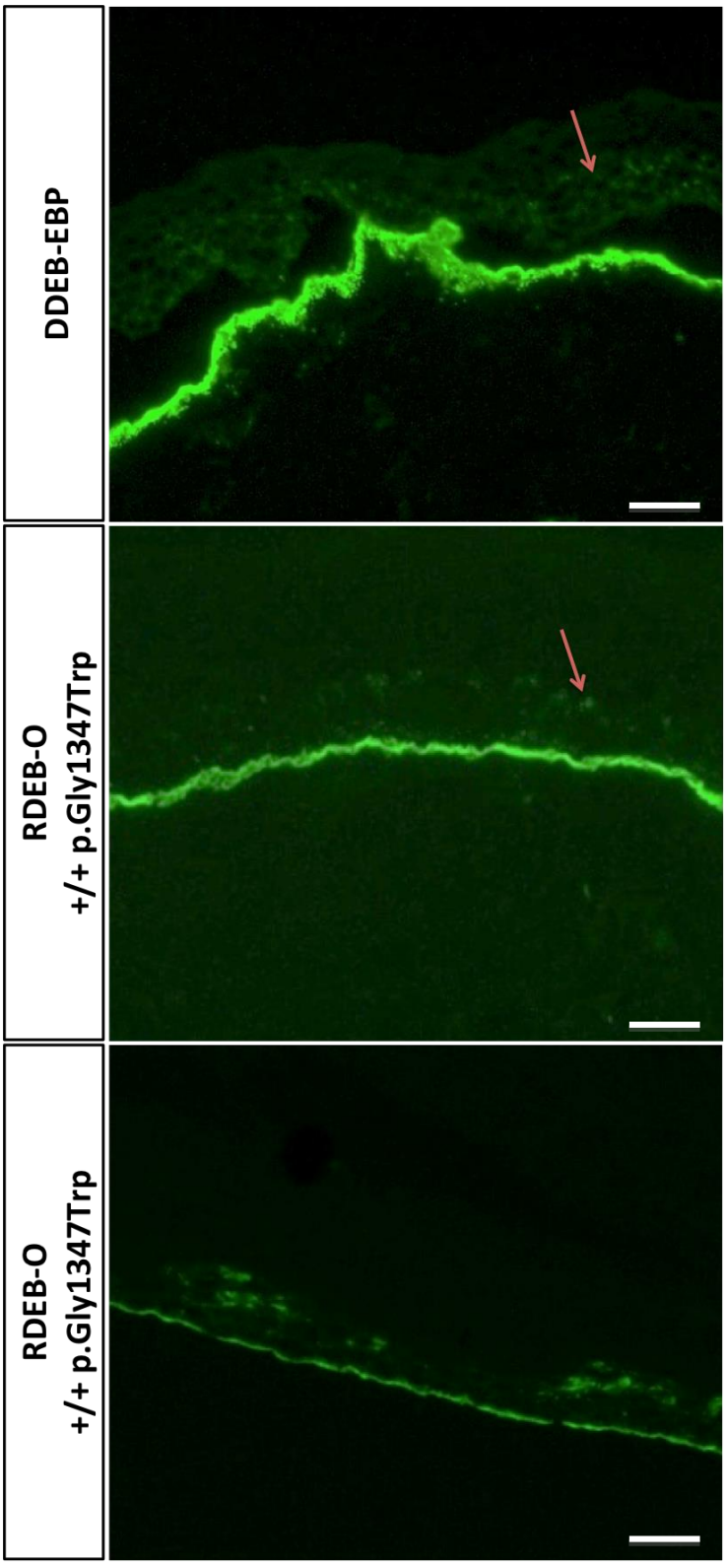
6	RDEB-O	*COL7A1*	p.Gly1907Asp/ IVS79+1G>C[a]	E68/ I79	MS/SS	AR	No	reduced, maps to roof of blister	focal basal/ suprabasal	ND	ND
7	RDEB-sev gen	*COL7A1*	**p.Arg137Stop**/ c.6269delC	E3/ E75	PTC/PTC	AR	No	absent labelling, split present	focal basal/ suprabasal	ND	ND
8	RDEB-sev gen	*COL7A1*	**p.Glu302Stop**/ p.Arg525Stop	E7/ E12	PTC/PTC	AR	No	reduced, maps to roof of blister	generalised basal/ suprabasal	reduced, wispy	atypical vesicular inclusion bodies in basal layer
9	RDEB-sev gen	*COL7A1*	p.Gly1770Ser/ p.Gly1770Ser[a]	E61	MS/MS	AR	No	reduced, maps to roof and base of blister	generalised basal/ suprabasal	reduced, wispy	atypical homogenous and granular inclusion bodies in basal and suprabasal layers. Dilated RER
10	RDEB-sev gen	*COL7A1*	p.Gly2369Ser/ Gly2369Ser[a]	E93	MS/MS	AR	No	absent labelling, split present	generalised basal	reduced	typical perinuclear granular stellate bodies in basal layer
Non-dystrophic epidermolysis bullosa											
11	EBS-DM	*KRT5*	p.Glu477Lys	E7	MS	AD*	No	normal, maps to base of blister	focal basal/ suprabasal	ND	ND
12	EBS-DM	*KRT14*	p.Asn123Ser	E1	MS	AD*	No	normal, maps to base of blister	generalised basal/ suprabasal	ND	ND

13	EBS-DM	*KRT14*	p.Arg125His	E1	MS	AD	No	normal, no blister	generalised basal/ suprabasal	slight variation in number and morphology	atypical granular inclusion bodies in basal layer. Dilated RER
14	EBS	*KRT14*	**c.1140-1170dup31**	E6	PTC	AD	No	normal, no blister	focal basal/ suprabasal	slight variation in number and morphology	atypical granular inclusion bodies
15	HJEB	*LAMA3*	**p.Gln1794Stop/ c.7888delG**	E43/E61	PTC/PTC	AR	No	normal, maps to base of blister	focal basal/ suprabasal	ND	ND
16	HJEB	*LAMA3*	**p.Trp3241Stop/ p.Trp3241Stop**	E74	PTC/PTC	AR	No	normal, maps to base of blister	focal basal/ suprabasal	ND	ND
17	nHJEB	*LAMB3*	**IVS8+1G>C**/ p.Arg635Stop	I8/E14	SS/PTC	AR	No	normal, maps to base of blister	generalised basal/ suprabasal	ND	ND
18	HJEB	*LAMB3*	c.3162delG/ c.3162delG	E21	PTC/PTC	AR	No	normal, maps to base of blister	focal basal/ suprabasal	ND	ND
19	aplasia cutis	ND	ND	ND	ND	ND	No	normal, no blister	generalised basal/ suprabasal	some variation in number and morphology	atypical granular inclusion bodies and empty vesicles in basal and suprabasal layers

20	unknown	ND	ND	ND	ND	ND	No	normal, no blister	focal basal/ suprabasal	slight variation in number and morphology	atypical granular inclusion bodies/empty vesicles in basal and suprabasal layers
21	unknown	ND	ND	ND	ND	ND	No	normal, no blister	focal basal/ suprabasal	some variation in morphology	atypical homogenous inclusion bodies in basal layer

bold, novel mutations; ND -not determined; EB, epidermolysis bullosa; DEB, dystrophic EB; DDEB, dominant DEB; RDEB, recessive DEB; EB-pr, EB pruriginosa; RDEB-O, RDEB other; RDEB-sev gen, RDEB severe generalised; EB-DM, EB Dowling Meara; EBS, EB simplex; HJEB, Herlitz junctional EB, nHJEB, non-Herlitz junctional EB; DEJ, dermal-epidermal junction; IVS, intervening sequence (intron); MS, missense; PTC, premature termination codon; RER, rough endoplasmic reticulum; SS, splice site; AD, autosomal dominant; AR, autosomal recessive. [a]Almaani *et al*. Acta Derm Venereol. 2011;91:262-6.

Figure 3.4 Immunohistochemical and ultrastructural appearance of non-BDN cases of DEB in which intra-epidermal collagen VII was detected in this study. Descriptive details of the DIF and TEM findings are presented in Table 3.4. Arrows highlight intra-epidermal collagen VII. Bars = 50 μm.



RDEB-O
+/+ p.Gly1483Asp
RDEB-O
(+/+) c.IVS55-1G>T
RDEB-O
p.Gly1907Asp/IVS79+1G>C

RDEB-sev gen
+/+ p.Gly1770Ser
RDEB-sev gen
p.Glu302Stop/p.Arg525Stop
RDEB-sev gen
p.Arg137Stop/c.6269delC

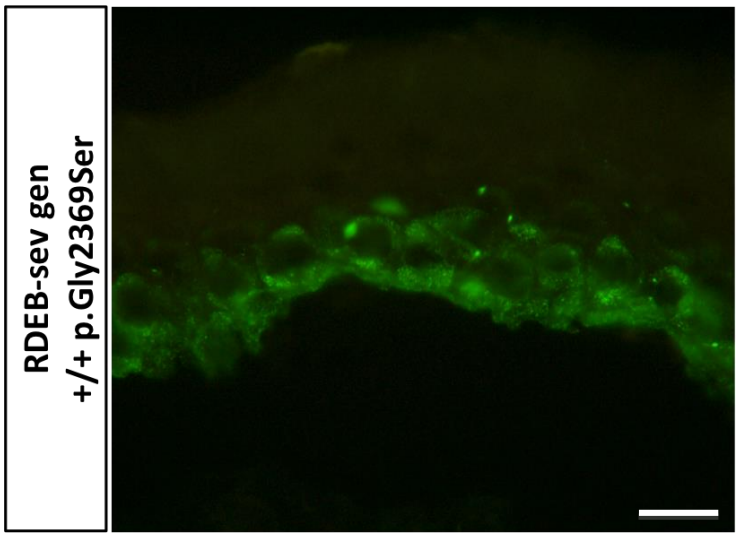
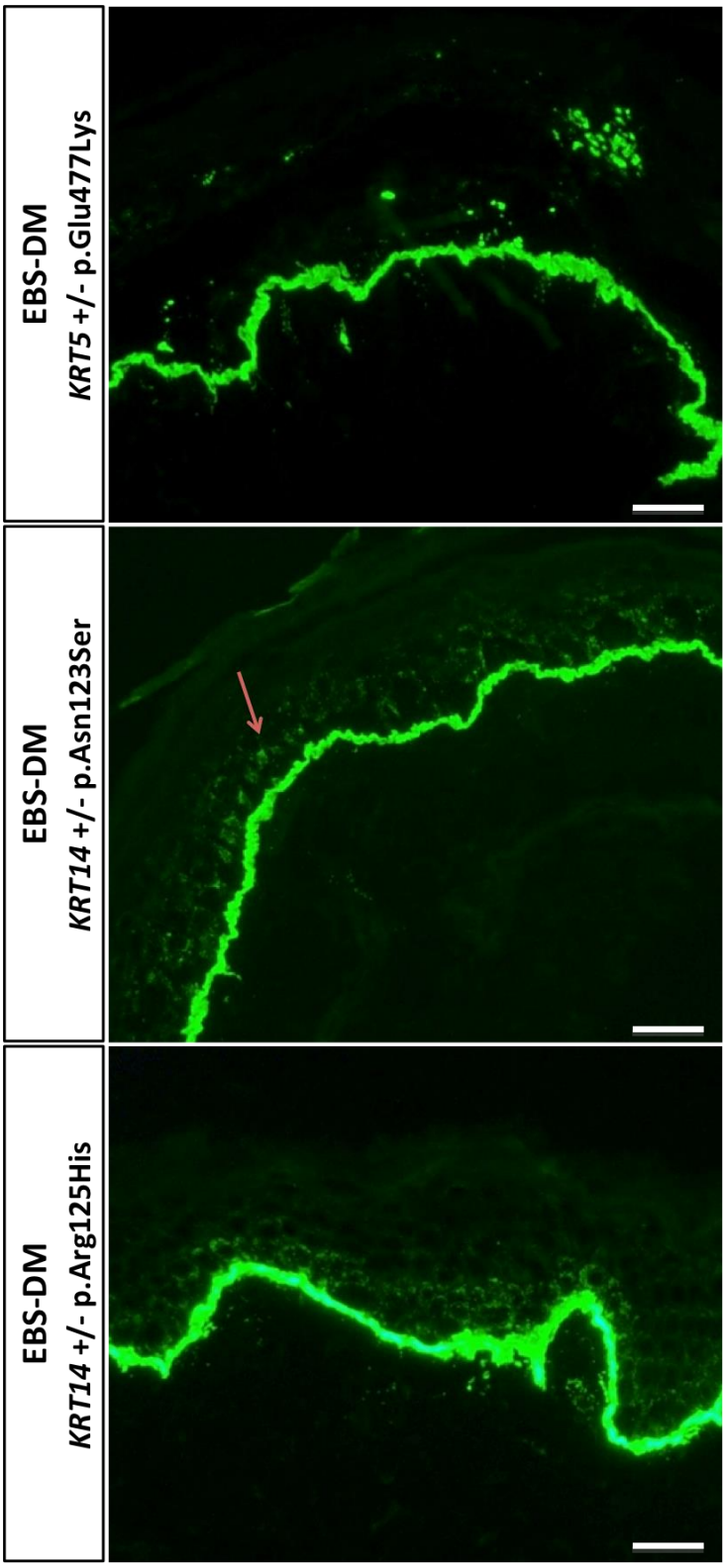
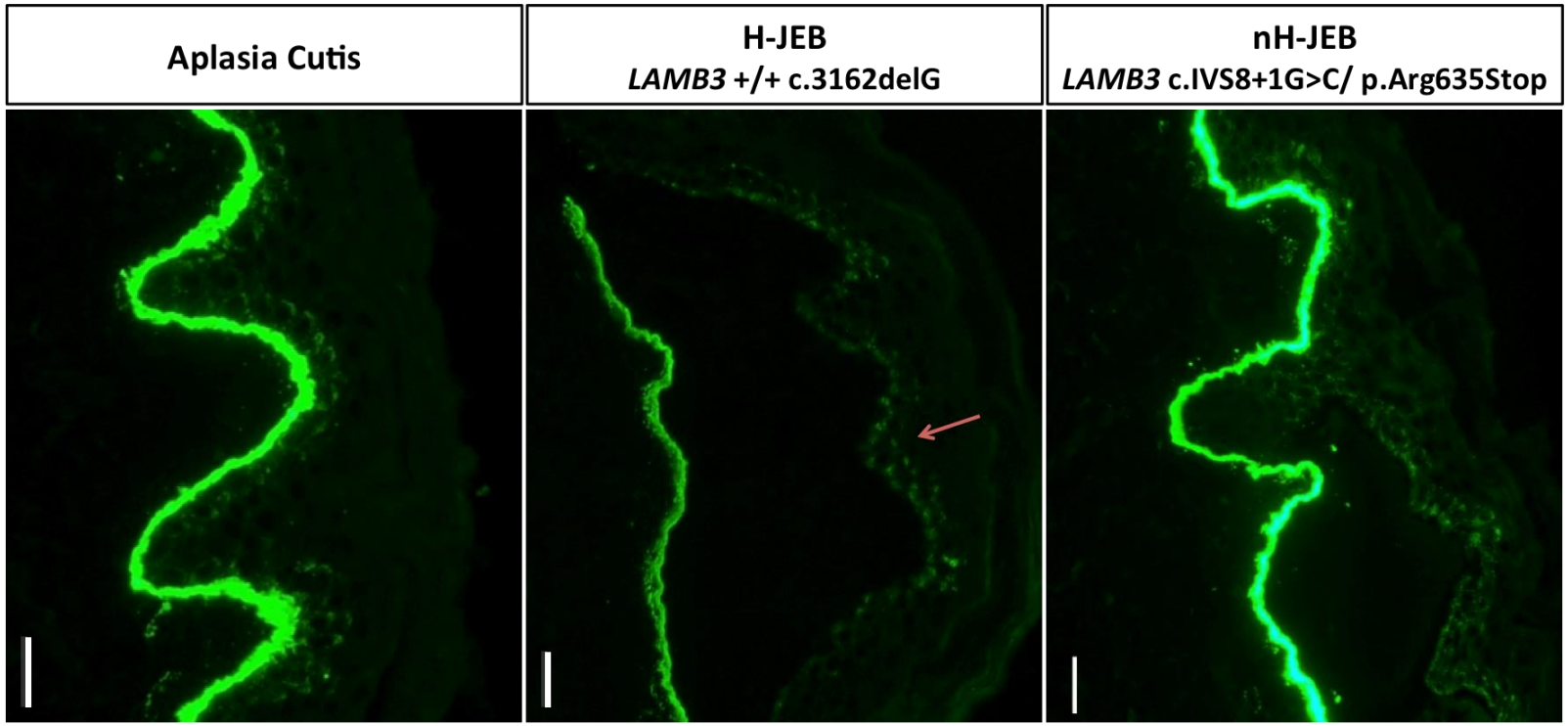

RDEB-sev gen
+/+ p.Gly2369Ser

Figure 3.5 Immunohistochemical and ultrastructural appearances of non-DEB cases that displayed intra-epidermal collagen VII in this study. Descriptive details of the DIF and TEM findings are presented in Table 3.4. Bars = 50 μm



H-JEB
LAMA3 +/+ p.Trp3241Stop
H-JEB
LAMA3 p.Gln1794Stop/c.7888delG
EBS
KRT14 +/- c.1140-1170dup31


Aplasia Cutis
H-JEB
LAMB3 +/+ c.3162delG
nH-JEB
LAMB3 c.IVS8+1G>C/ p.Arg635Stop

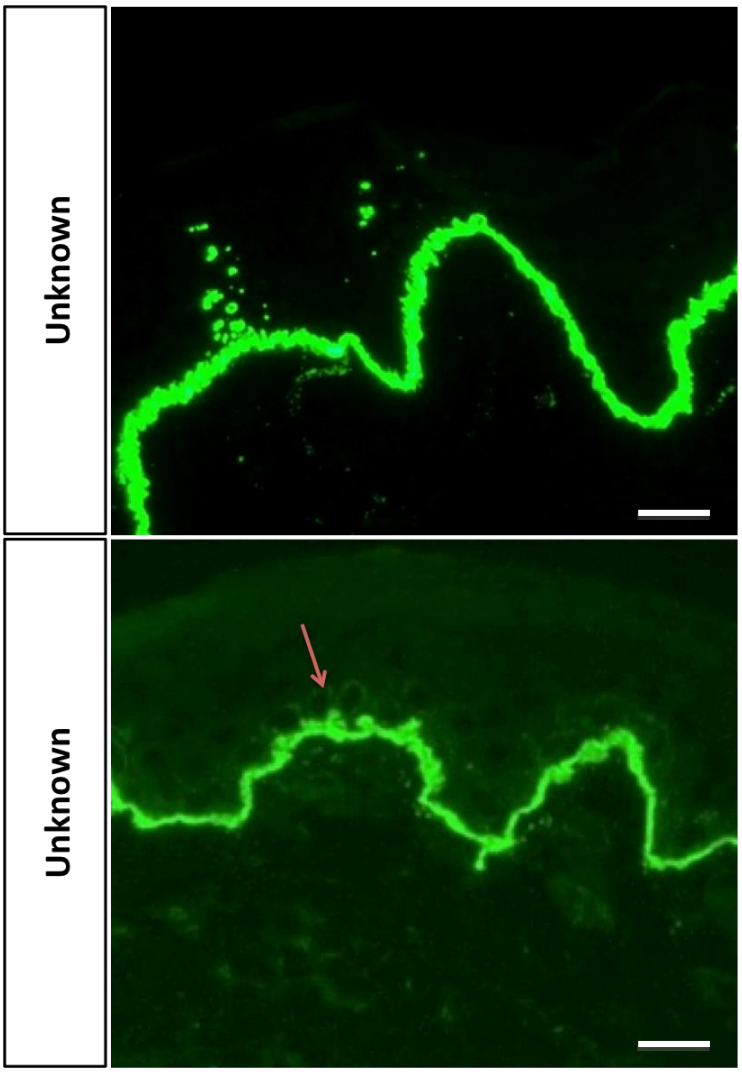
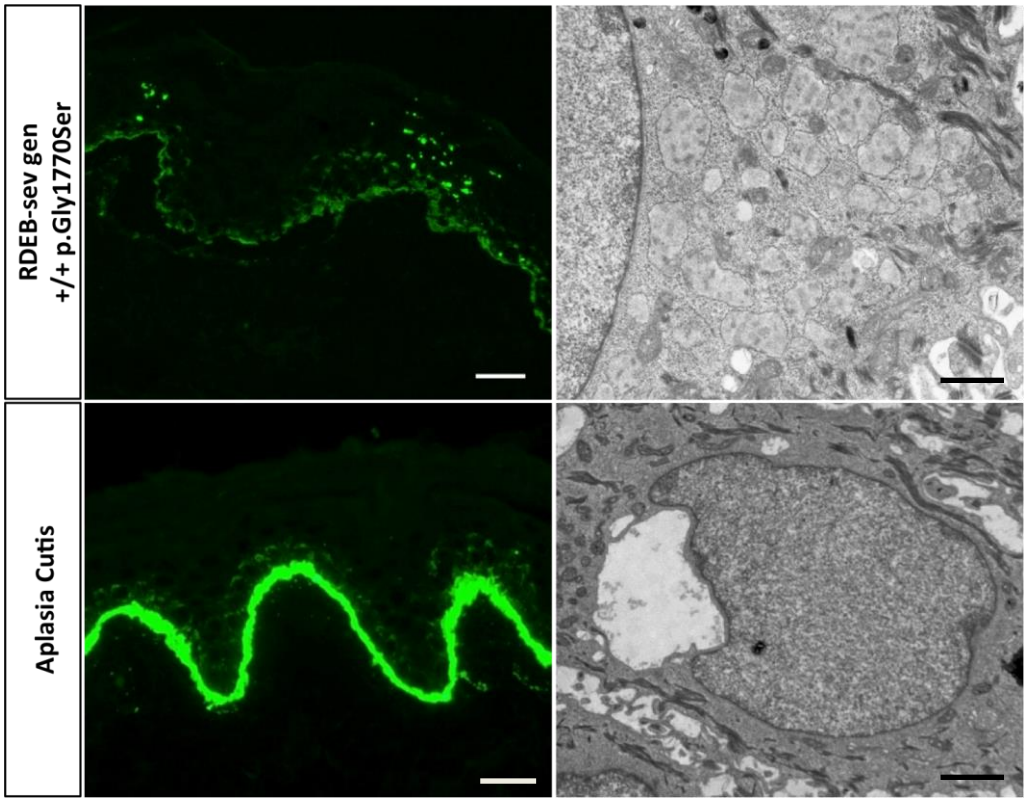

Unknown
Unknown

Figure 3.6 Immunofluorescence and ultrastructural findings in 2 non-BDN cases with intracytoplasmic retention of collagen VII. (A) In a case of RDEB-sev gen, basal and suprabasal retention of collagen VII is demonstrated on DIF. This is associated with typical perinuclear stellate bodies on TEM. (B) A case of aplasia cutis with intraepidermal retention of collagen VII on DIF and a large granular stellate body on TEM. This highlights that the presence of intracytoplasmic retention within the epidermis could occur in cases unrelated to collagen VII pathology. IMF bars = 50 μm; TEM bars = 2.5 μm.



3.4 Discussion

Although most reported cases of BDN improve clinically, not all cases demonstrate complete resolution of skin fragility, prompting the change of the original nomenclature from TBDN to BDN (Fine *et al.*, 2008; Fine *et al.*, 1990). The data outlined above supports this, as 3 out of 14 cases of BDN did not show complete resolution of skin blistering. Moreover, although intracytoplasmic retention of collagen VII is classically associated with BDN, this study demonstrates that intra-epidermal collagen VII is not exclusive to cases of BDN, which only represented about one-third of cases in the studied cohort. In addition to the previously reported examples of non-BDN associated intra-epidermal collagen VII, this IF pattern was also noted in cases of EB simplex, junctional EB and 1 case of aplasia cutis. Precisely why this IF finding occurred in these cases, all of which lacked any primary pathology in collagen VII, is unclear although disruption of hemidesmosomes and/or the cytoskeleton could conceivably lead to secondary impairment of collagen VII transport and secretion from keratinocytes to basement membrane and, indeed, some degree of intra-epidermal collagen VII within basal keratinocytes has been noted in the majority of cases of JEB in which hemidesmosomes are fewer and hypoplastic (R. Eady and J. McGrath unpublished observations). It is worth noting that some technical and chronological factors might contribute to the presence or absence of intracytoplasmic collagen VII, including the stage at which the biopsy was taken, as well as, gestational age. Samples taken at a later stage of wound evolution and at an older gestational age are likely to be associated with a reduced expression of collagen VII perhaps relating to restoration of collagen VII expression as it is eventually secreted into the DEJ.

At a molecular level, intracytoplasmic accumulation of collagen VII has been largely associated with missense substitution mutations in the triple helical domain of collagen VII (Tables 3.1 and 3.2) (Hammami-Hauasli *et al.*, 1998b; Jonkman *et al.*, 1999; Chen *et al.*, 2002a; Sawamura *et al.*, 2006a; Nakano *et al.*, 2007). Notably, the missense mutations p.Gly2006Asp, p.Gly2034Arg, p.Gly2015Glu (Hammami-Hauasli *et al.*, 1998b) and p.Arg2008Gly (Chen *et al.*, 2002a) in exon 73 have been shown to interfere with collagen VII folding and secretion. In addition, confocal laser studies and semiquantitative immunoblotting determined that DEB keratinocytes retained up to 2.5-fold more procollagen VII within the rough endoplasmic reticulum than controls causing intracellular accumulation of mutant collagen VII molecules (Hammami-Hauasli *et al.*, 1998b). This was also supported by in vitro studies demonstrating the effects of certain missense mutations on type VII procollagen assembly (p.Arg2622Gln and p.Gly2623Cys) (Brittingham *et al.*, 2005) and secretion (p.Arg2008Gly) (Chen *et al.*, 2002a). In this study, cases with intracytoplasmic retention of collagen VII generally and BDN specifically demonstrated genetic heterogeneity (Table 3.3). Mutational analysis was performed on 30 out of 35 cases. Heterozygous mutations were demonstrated in 5 patients with BDN: p.Gly1522Glu, c.2021insG and IVS73-3del6, illustrating a missense glycine substitution mutation, loss of function mutation and a splice site mutation respectively. 3 cases demonstrated recessive inheritance: p.Arg2008His/ p.Arg2008His, p.Gly2781Ser/ p.Arg1632Stop and p.Gly2372Val/ p.Gly2372Val. The majority of DEB cases with intracytoplasmic retention of collagen VII were due to heterozygous or homozygous GS mutations. However, there was no demonstrable genotype-

phenotype correlation based on the nature and location of the GS mutations. With regards to the non-DEB cases, the clinical picture and underlying mutations were quite variable and the number of patients was too small to make a distinct correlation.

However, I acknowledge that due to the retrospective nature of this study, detailed clinical information was not available for all cases resulting an element of ascertainment bias, for example, a case of mild-RDEB that could represent RDEB-BDN if followed up for a prolonged period of time. Longitudinal studies would overcome this limitation and would provide a more accurate measure of clinical outcome and genotype-phenotype correlation. Furthermore, BDN cases included in this study were selected retrospectively based on the presence of the intracytoplasmic collagen VII. This selection might have excluded genuine cases of BDN that lacked intraepidermal retention of collagen VII due to various reasons; including the age and wound stage at the time of skin sampling.

Ultrastructurally, most reports of intracytoplasmic retention of collagen VII describe anchoring fibrils with reduced number and abnormal morphology, in addition to dilated RER. The stellate body which has been used as a proxy for BDN refers to dilated RER which have fused to form single cystic spaces or vacuoles containing electron dense amorphous material shown to represent collagen VII molecules (Hashimoto *et al.*, 1985; Hashimoto *et al.*, 1989; Fine *et al.*, 1990). In this study the ultrastructural findings were quite variable. All cases showed some degree of RER dilatation particularly in basal keratinocytes. However, the nature of the intracytoplasmic bodies ranged from empty vesicles, homogenous inclusion bodies and the classical granular stellate

bodies. Although a few non-BDN cases showed some structures similar to the classical stellate bodies (Figure 3.6), this was only observed in cases of BDN, whereas the more homogenous inclusion bodies were observed in all other cases including BDN and normal skin and therefore, their significance is yet to be determined. Therefore, our findings show that in cases of intracytoplasmic retention of collagen VII, the presence of the typical stellate body represents a consistent discriminator between cases of BDN and non-BDN cases. However, its absence does not rule out BDN.

Thus, the definition of the stellate body, which has been used synonymously with BDN, might be somewhat confusing and perhaps should be referred to as a variant of dilated RER. Furthermore, although TEM is a useful tool in assessing intra cytoplasmic retention of collagen VII, it is not widely available and requires experience and close analysis to visualize the classical stellate body. Numerous semithin sections are usually required to visualize these bodies and TEM correlation with IF would still be performed in all cases. In addition, other factors might influence the presence and abundance of intracytoplasmic inclusion bodies immunohistochemically and ultrastructurally including the time at which the biopsy was taken in relation to the onset of skin fragility, wound healing and technical factors relating to tissue staining.

In conclusion, intracytoplasmic retention of collagen VII is not exclusive to cases of BDN and can be associated with DDEB, RDEB, JEB, EBS as well as normal skin. Thus the finding of intra-epidermal retention of collagen VII can have diverse diagnostic, phenotypic and prognostic implications. Immunofluorescence and electron microscopy studies can

potentially provide insight into future clinical outcome. In particular, the presence of the classical stellate body seems to represent a good discriminator between BDN and non-BDN cases as evidenced by its presence in BDN cases only. However, definitive diagnosis will always require molecular mutational analysis and prospective clinical assessments.

Chapter 4

The spectrum of dominant and recessive glycine substitution mutations in *COL7A1*

Abstract

DEB results from mutations in *COL7A1* leading to structural or numerical disruption of anchoring fibrils at the basement membrane zone. This leads to loss of dermo-epidermal integrity with blistering beneath the lamina densa. Dominant forms of DEB usually result from heterozygous (dominant-negative) glycine substitution (GS) mutations within the collagen VII triple helix. In contrast, recessive DEB is a consequence of nonsense, frameshift or splice site mutations on both *COL7A1* alleles. However, some cases of recessive DEB also involve GS mutations which are usually silent when inherited on one allele, but pathogenic when inherited on both alleles or *in trans* with another loss-of-function mutation. This poses a challenge when trying to differentiate between dominant and recessive cases of DEB with important implications on diagnosis and genetic counselling. In addition, some unusual phenotype-genotype patterns add to the diagnostic dilemmas including some GS mutations resulting in both dominant and recessive DEB. Also, the same GS mutation can result in variable disease expression that can range from normal skin, to nail dystrophy alone or widespread skin fragility. In this study, I report 25 novel dominant and 15 novel recessive *COL7A1* GS mutations underlying DEB, including 4 that result in both dominant and recessive disease. These findings highlight phenotypic and genotypic heterogeneity important for establishing accurate paradigms for genotype-phenotype correlation.

4.1 Introduction

Fibrillar and non-fibrillar collagens are formed of 3 distinct α-polypeptide chains that are characterised by a high glycine index due to a repeated Gly-*X*-*Y* triplet sequence within the central collagenous domain. This sequence is uninterrupted in fibrillar collagens such as collagen I, whereas there are interruptions within non-fibrillar collagens including collagen VII that confer flexibility to the suprastructural conformation but also increase its susceptibility for non-specific protease degradation. As mentioned in Chapter 1, the collagen VII central collagenous domain contains 19 non-collagenous interruptions, including the 'hinge region', which is thought to confer flexibility necessary for collagen VII suprastructural folding (Christiano *et al.*, 1994c; Christiano *et al.*, 1994b; Chung and Uitto, 2010). The central collagenous domain is flanked by the NC1 domain, which is thought to affect collagen VII attachment to the BMZ (Chen *et al.*, 2001) and the NC2 domain, that contains highly conserved cysteine residues and has been found to initiate the assembly of collagen VII triple helices and formation of anti-parallel dimers (Chen *et al.*, 2001).

Integral to the collagen VII suprastructure is glycine, the smallest amino acid that is positioned towards the core of the triple helix, whereas the X and Y motifs are aligned at the surface of the helical collagen VII structure. As a result, any disruption to the glycine residue or substitution with a bulkier amino acid is likely to result in abnormal triple-helical conformation and subsequent clinical pathology (Prockop and Kivirikko, 1995; Myllyharju and Kivirikko, 2004; Myllyharju and Kivirikko, 2001; Boot-Handford and Tuckwell, 2003). This is supported by results showing that any type of GS is

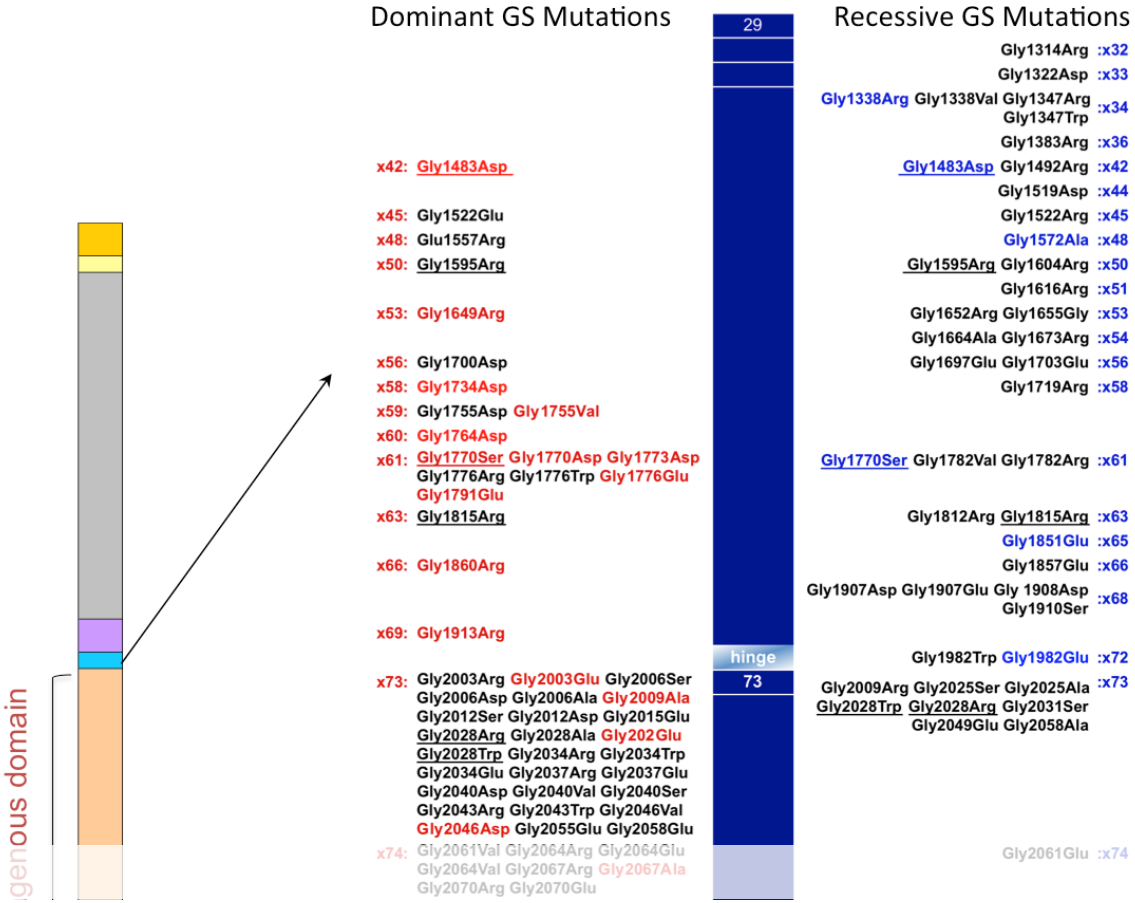
capable of causing disease (Persikov *et al.*, 2004), reflecting the disruptive and destabilising effects on the collagen VII triple helix as well as the extent of evolutionary conservation at the Gly positions (Kivirikko *et al.*, 1996). However, although any GS mutation can cause pathology, there is vast genotype and phenotype heterogeneity.

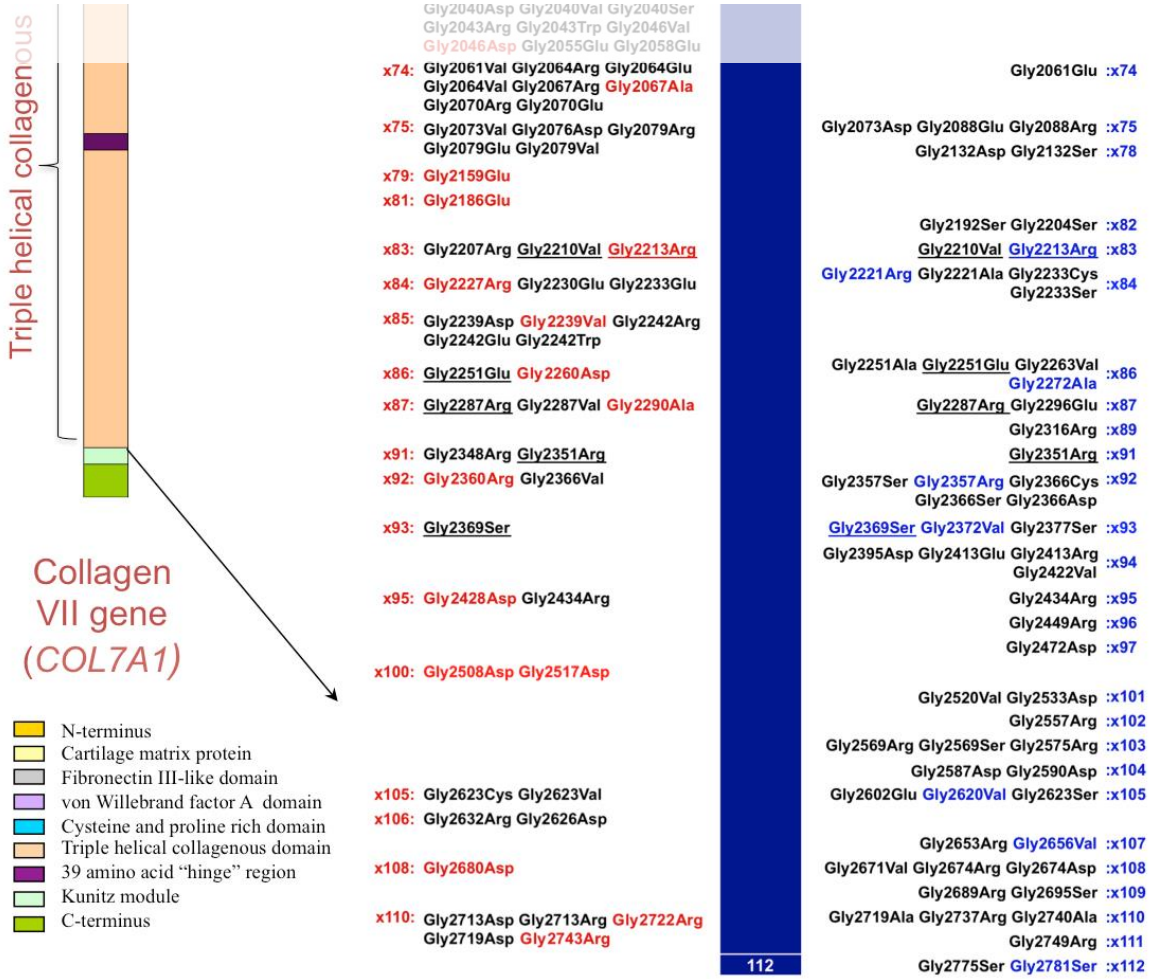
Around 140 *COL7A1* GS mutations have been reported in the literature (Figure 4.1)**.** Dominant forms of DEB usually result from heterozygous (dominant-negative) GS mutations within the collagen VII triple helix. In contrast, recessive DEB is a consequence of nonsense, frameshift or splice site mutations on both *COL7A1* alleles (Uitto *et al.*, 1994; Whittock *et al.*, 1999; Dang and Murrell, 2008) but can result from GS mutations which are usually silent when inherited on one allele, but pathogenic when inherited on both alleles or *in trans* with another loss-of-function mutation (Shimizu *et al.*, 1996; Uitto *et al.*, 1994). In addition, some unusual phenotype-genotype patterns add to the diagnostic dilemmas including some GS mutations resulting in both dominant and recessive DEB (Christiano *et al.*, 1996a; Hammami-Hauasli *et al.*, 1998a; Shimizu *et al.*, 1999; Lee *et al.*, 2000; Sato-Matsumura *et al.*, 2002; Varki *et al.*, 2007; Murata *et al.*, 2000; Nakamura *et al.*, 2004; Dang *et al.*, 2007; Sambrook *et al.*, 2001; Christiano *et al.*, 1997b). Furthermore, the same GS mutation can result in a variable disease expression that can range from normal skin or nail dystrophy alone to widespread skin fragility (Lee *et al.*, 2000; Murata *et al.*, 2000; Varki *et al.*, 2007).

The aim of this Chapter is to explore the nature of GS mutations in the collagen VII gene, their functional effects as well as potential factors

influencing disease expression in an aim to refine genotype-phenotype correlation.

Figure 4.1 A schematic outlining the position of GS mutations within the *COL7A1* triple helix. Novel dominant and recessive mutations identified in this study are highlighted in red and blue respectively. Mutations that result in both dominant and recessive disease are underlined in black. The triple helical domain is indicated by the arrows.




Triple helical collagenous
Collagen VII gene (COL7A1)
N-terminus
Cartilage matrix protein
Fibronectin III-like domain
von Willebrand factor A domain
Cysteine and proline rich domain
Triple helical collagenous domain
39 amino acid "hinge" region
Kunitz module
C-terminus
Gly2040Asp Gly2040Val Gly2040Ser
Gly2043Arg Gly2043Trp Gly2046Val
Gly2046Asp Gly2055Glu Gly2058Glu
x74: Gly2061Val Gly2064Arg Gly2064Glu Gly2064Val Gly2067Arg Gly2067Ala Gly2070Arg Gly2070Glu
x75: Gly2073Val Gly2076Asp Gly2079Arg Gly2079Glu Gly2079Val
x79: Gly2159Glu
x81: Gly2186Glu
x83: Gly2207Arg Gly2210Val Gly2213Arg
x84: Gly2227Arg Gly2230Glu Gly2233Glu
x85: Gly2239Asp Gly2239Val Gly2242Arg Gly2242Glu Gly2242Trp
x86: Gly2251Glu Gly2260Asp
x87: Gly2287Arg Gly2287Val Gly2290Ala
x91: Gly2348Arg Gly2351Arg
x92: Gly2360Arg Gly2366Val
x93: Gly2369Ser
x95: Gly2428Asp Gly2434Arg
x100: Gly2508Asp Gly2517Asp
x105: Gly2623Cys Gly2623Val
x106: Gly2632Arg Gly2626Asp
x108: Gly2680Asp
x110: Gly2713Asp Gly2713Arg Gly2722Arg Gly2719Asp Gly2743Arg
112
Gly2061Glu :x74
Gly2073Asp Gly2088Glu Gly2088Arg :x75
Gly2132Asp Gly2132Ser :x78
Gly2192Ser Gly2204Ser :x82
Gly2210Val Gly2213Arg :x83
Gly2221Arg Gly2221Ala Gly2233Cys Gly2233Ser :x84
Gly2251Ala Gly2251Glu Gly2263Val Gly2272Ala :x86
Gly2287Arg Gly2296Glu :x87
Gly2316Arg :x89
Gly2351Arg :x91
Gly2357Ser Gly2357Arg Gly2366Cys Gly2366Ser Gly2366Asp :x92
Gly2369Ser Gly2372Val Gly2377Ser :x93
Gly2395Asp Gly2413Glu Gly2413Arg Gly2422Val :x94
Gly2434Arg :x95
Gly2449Arg :x96
Gly2472Asp :x97
Gly2520Val Gly2533Asp :x101
Gly2557Arg :x102
Gly2569Arg Gly2569Ser Gly2575Arg :x103
Gly2587Asp Gly2590Asp :x104
Gly2602Glu Gly2620Val Gly2623Ser :x105
Gly2653Arg Gly2656Val :x107
Gly2671Val Gly2674Arg Gly2674Asp :x108
Gly2689Arg Gly2695Ser :x109
Gly2719Ala Gly2737Arg Gly2740Ala :x110
Gly2749Arg :x111
Gly2775Ser Gly2781Ser :x112

4.2 Methods

For this study I collected and evaluated DNA mutation analysis data retrospectively, via the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory database in London. Blood samples from patients with clinically suspected or histologically proven DEB (and their parents if available), were provided historically by local, national and international referrers. Patient ethnicities included white Caucasian, Middle-Eastern, South American, South-East Asian and Asian. Prior to my work on this thesis, the DNA extraction and *COL7A1* mutation analysis had been performed by Dr. Lu Liu at the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory, as part of the patients' routine clinical care in most cases. However, for individuals linked to separate research projects, DNA screening was undertaken with Ethics' Committee approval (St Thomas' Hospital Ethics' committee; 07/H0802/104) with informed consent and carried out in accordance with the Declaration of Helsinki principles. DNA extraction and sequencing was performed as discussed in Chapter 2.

4.3 Results

The *COL7A1* gene was sequenced in >200 individuals with different forms of DEB leading to the identification of 46 new glycine substitution mutations resulting in either dominant DEB (n=31; p.Gly1483Asp, p.Gly1649Arg, p.Gly1734Asp, p.Gly1755Val, p.Gly1764Asp, p.Gly1770Ser, p.Gly1770Asp, p.Gly1773Asp, p.Gly1776Glu, p.Gly1860Arg, p.Gly1913Arg, p.Gly2003Glu, p.Gly2009Ala, p.Gly2028Glu, p.Gly2046Asp, p.Gly2067Ala,

p.Gly2159Glu, p.Gly2186Glu, p.Gly2213Arg, p.Gly2227Arg, p.Gly2233Glu, p.Gly2239Val, p.Gly2260Asp, p.Gly2290Ala, p.Gly2360Arg, p.Gly2428Asp, p.Gly2508Asp, p.Gly2517Asp, p.Gly2680Asp, p.Gly2722Arg, p.Gly2743Arg) (Table 4.1) or recessive DEB (n=15; p.Gly174Arg, p.Gly1338Arg, p.Gly1483Asp, p.Gly1572Ala, p.Gly1770Ser, p.Gly1851Glu, p.Gly1940Asp, p.Gly2213Arg, p. Gly2221Arg, p.Gly2272Ala, p.Gly2369Ser, p.Gly2372Val, p.Gly2375Ser, p.Gly2620Val, p. Gly2781Arg) (Table 4.2).

Four of these novel GS mutations in *COL7A1* (p.Gly1483Asp, p.Gly1770Ser, p.Gly2213Arg and p.Gly2369Ser) were also noted to function in a dominant or recessive manner (Figure 4.2).

Table 4.1 Novel and reported dominant GS mutations in *COL7A1*.

Diagnosis	Exon	Glycine Substitution	cDNA	Reference
DDEB-BDN	42	**p.Gly1483Asp**	c.4448G>A	**This Study**
DDEB	45	p.Gly1522Glu	c.4565G>A	(Whittock *et al.*, 1999)
DDEB-gen	48	p.Gly1557Arg	c.4669G>A	(Christiano *et al.*, 1996a)
DDEB-na	50	p.Gly1595Arg	c.4783G>C	(Sato-Matsumura *et al.*, 2002)
DDEB-gen	53	**p.Gly1649Arg**	c.4945G>C	**This Study**
DDEB-pr DDEB-gen	56	p.Gly1700Asp	c.5099G>A	(Deng *et al.*, 2008)
DDEB-gen	58	**p.Gly1734Asp**	c.5201G>A	**This Study**
DDEB-pr	59	p.Gly1755Asp*	c.5264G>C	(Posteraro *et al.*, 2005)
DDEB-gen	59	**p.Gly1755Val**	c.5264G>T	**This Study**
DDEB-gen	60	**p.Gly1764Asp**	c.5291G>A	**This Study**
DDEB-na/ DDEB-pt	61	**p.Gly1770Ser**	c.5308G>A	**This Study**
DDEB-pr	61	**p.Gly1770Asp**	c.5309G>A	**This Study**
DDEB-gen	61	**p.Gly1773Asp**	c.5318G>A	**This Study**
DDEB	61	p.Gly1776Arg	c.5326G>A	(Whittock *et al.*, 1999)
DDEB-gen	61	p.Gly1776Trp*	c.5326G>T	(Bursztejn *et al.*, 2008)
DDEB-gen	61	**p.Gly1776Glu**	c.5327G>A	**This Study**
DDEB-pr	61	p.Gly1791Glu	c.5372G>A	(Mellerio *et al.*, 1999)

DDEB-na	63	p.Gly1815Arg	c.5443G>A	(Sato-Matsumura *et al.*, 2002)
DDEB-pr	66	**p.Gly1860Arg**	c.5578G>A	**This Study**
DDEB-pr	69	**p.Gly1913Arg**	c.5737G>C	**This Study**
DDEB-gen	73	p.Gly2003Arg	c.6007G>A	(Christiano *et al.*, 1996b)
DDEB-gen	73	**p.Gly2003Glu**	c.6008G>A	**This Study**
DDEB-gen	73	p.Gly2006Ser*	c.6016G>A	(Mallipeddi *et al.*, 2003)
DDEB-gen	73	p.Gly2006Asp	c.6017G>A	(Hammami-Hauasli *et al.*, 1998b)
DDEB	73	p.Gly2006Ala	c.6018G>C	(Whittock *et al.*, 1999)
DDEB-gen	73	**p.Gly2009Ala**	c.6026G>C	**This Study**
DDEB	73	p.Gly2012Ser*	c.6034G>A	(Gardella *et al.*, 2002)
DDEB-gen	73	p.Gly2012Asp*	c.6035G>A	(Matsuba *et al.*, 2002)
DDEB-gen	73	p.Gly2015Glu	c.6044G>A	(Hammami-Hauasli *et al.*, 1998b)
DDEB-gen	73	p.Gly2028Arg	c.6082G>A	(Lee *et al.*, 2000)
DDEB-gen	73	p.Gly2028Trp	c.6082G>T	(Varki *et al.*, 2007)
DDEB-gen	73	p.Gly2028Ala	c.6083G>C	(Murata *et al.*, 2000)
DDEB-gen	73	**p.Gly2028Glu**	c.6083G>A	**This Study**
DDEB-gen	73	p.Gly2034Arg	c.6100G>A	(Kon *et al.*, 1997a)
DDEB-gen	73	p.Gly2034Trp	c.6100G>T	(Rouan *et al.*, 1998)
DDEB	73	p.Gly2034Glu	c.6101G>A	(Kern *et al.*, 2006)
DDEB-gen	73	p.Gly2037Arg	c.6109G>A	(Iwata *et al.*, 2006)
DDEB-gen	73	p.Gly2037Glu	c.6110G>A	(Jonkman *et al.*, 1999)

DDEB-gen	73	p.Gly2040Ser	c.6118G>A	(Christiano *et al.*, 1994d)
DDEB	73	p.Gly2040Asp	c.6119G>A	(Whittock *et al.*, 1999)
DDEB-gen	73	p.Gly2040Val*	c.6119G>T	(Rouan *et al.*, 1998)
DDEB	73	p.Gly2043Arg*	c.6127G>A	(Christiano *et al.*, 1995b)
DDEB-gen	73	p.Gly2043Trp*	c.6127G>T	(Mecklenbeck *et al.*, 1999)
DDEB	73	p.Gly2046Val	c.6137G>T	(Whittock *et al.*, 1999)
DDEB-gen	73	**p.Gly2046Asp**	c.6137G>A	**This Study**
DDEB-gen	73	p.Gly2055Glu	c.6164G>A	(Christiano *et al.*, 1996a)
DDEB-pt	73	p.Gly2059Glu[a]	c.6137G>A[b]	(Hamada *et al.*, 2009)
DDEB-gen	74	p.Gly2061Val	c.6182G>T	(Escamez *et al.*, 2010)
DDEB-gen	74	p.Gly2064Arg	c.6190G>A	(Rouan *et al.*, 1998)
DDEB-gen	74	p.Gly2064Val	c.6191G>T	(Kern *et al.*, 2009a)
DDEB	74	p.Gly2064Glu	c.6191G>A	(Sawamura *et al.*, 2005)
DDEB	74	p.Gly2067Arg*	c.6199G>A	(Posteraro *et al.*, 2005)
DDEB-gen	74	**p.Gly2067Ala**	c.6200G>C	**This Study**
DDEB-gen	74	p.Gly2070Arg	c.6208G>A	(Zhang *et al.*, 2003)
DDEB	74	p.Gly2070Glu	c.6209G>A	(Gardella *et al.*, 2002)
DDEB-pr	75	p.Gly2073Val	c.6218G>T	(Drera *et al.*, 2006)
DDEB-gen	75	p.Gly2076Asp*	c.6227G>A	(Kon *et al.*, 1997a)
DDEB	75	p.Gly2079Arg	c.6235G>A	(Christiano *et al.*, 1999)
DDEB-gen	75	p.Gly2079Glu*	c.6236G>A	(Kon *et al.*, 1997a)

DDEB-gen	75	p.Gly2079Val	c.6236G>T	(Kern *et al.*, 2009a)
DDEB-pr	79	**p.Gly2159Glu**	c.6476G>A	**This Study**
DDEB-BDN	81	**p.Gly2186Glu**	c.6557G>A	**This Study**
DDEB-gen	83	p.Gly2207Arg	c.6619G>A	(Kon *et al.*, 1997a)
DDEB-gen	83	p.Gly2210Val	c.6629G>T	(Dang *et al.*, 2007)
DDEB-pr	83	**p.Gly2213Arg**	c.6637G>A	**This Study**
DDEB-gen	84	**p.Gly2227Arg**	c.6679G>A	**This Study**
DDEB-gen	84	p.Gly2230Glu	c.6689G>A	(Kern *et al.*, 2009a)
DDEB-pr	84	**p.Gly2233Glu**	c.6698G>A	**This Study**
DDEB-pr	85	p.Gly2239Asp	c.6716G>A	(Tamai *et al.*, 1998)
DDEB-pr	85	**p.Gly2239Val**	c.6716G>T	**This Study**
DDEB-pr	85	p.Gly2242Arg	c.6724G>A	(Lee *et al.*, 1997)
DDEB-pr	85	p.Gly2242Trp	c.6724G>T	(Shi and Feng, 2009)
DDEB-pr	85	p.Gly2242Glu	c.6725G>A	(Tamai *et al.*, 1998)
DDEB-na	86	p.Gly2251Glu	c.6752G>A	(Hammami-Hauasli *et al.*, 1998a)
DDEB-gen	86	**p.Gly2260Asp**	c.6779G>A	**This Study**
DDEB-na	87	p.Gly2287Arg	c.6859G>A	(Shimizu *et al.*, 1999)
DDEB	87	p.Gly2287Val	c.6860G>T	(Posteraro *et al.*, 2005)
DDEB-pr	87	**p.Gly2290Ala**	c.6869G>C	**This Study**
DDEB-gen	91	p.Gly2347Arg *	c.7042G>C	(Cserhalmi-Friedman *et al.*, 1999)
DDEB-gen	91	p.Gly2351Arg	c.7051G>A	(Christiano *et al.*, 1996a)

DDEB-pr	92	**p.Gly2360Arg**	c.7078G>A	**This Study**
DDEB-pr	92	p.Gly2366Val	c.7097G>T	(Chuang *et al.*, 2004)
DDEB-pr	93	p.Gly2369Ser	c.7105G>A	(Mellerio *et al.*, 1999)
DDEB-gen	95	**p.Gly2428Asp***	c.7283G>A	**This Study**
DDEB-gen	95	p.Gly2434Arg	c.7300G>A	(Cuadrado-Corrales *et al.*, 2009)
DDEB-pr	100	**p.Gly2508Asp**	c.7523G>A	**This Study**
DDEB-pr	100	**p.Gly2517Asp**	c.7550G>A	**This Study**
DDEB-pt	105	p.Gly2623Cys	c.7867G>T	(Christiano *et al.*, 1995a)
DDEB-pr	105	p.Gly2623Val*	c.7868G>T	(Schumann *et al.*, 2008)
DDEB-pr	106	p.Gly2626Asp	c.7877G>A	(Wang *et al.*, 2007)
DDEB	106	p.Gly2632Arg	c.7894G>A	(Posteraro *et al.*, 2005)
DDEB-pr	108	**p.Gly2680Asp**	c.8039G>A	**This Study**
DDEB-pr	110	p.Gly2713Arg	c.8137G>C	(Mellerio *et al.*, 1999)
DDEB-gen	110	p.Gly2713Asp	c.8138G>A	(Rouan *et al.*, 1998)
DDEB-pr	110	p.Gly2719Asp	c.8156G>A	(Riedl *et al.*, 2009)
DDEB-gen	110	**p.Gly2722Arg**	c.8164G>C	**This Study**
DDEB-gen	110	**p.Gly2743Arg**	c.8227G>C	**This Study**

DDEB: dominant dystrophic epidermolysis bullosa; DDEB-BDN: bullous dermolysis of the newborn; DDEB-gen: generalized; DDEB-na: nail atrophy; DDEB-pr: pruriginosa; DDEB-pt: DDEB-pretibial; MS: missense; bold: new mutations. * de novo mutations. [a]p.Gly2059Glu, [b]c.6137G>A but corrected nomenclature should be: p.Gly2058Glu, c.6173G>A respectively.

Table 4.2 Novel and reported recessive GS mutations in the collagen VII gene.

Diagnosis	Exon	Allele 1 Glycine Substitution	cDNA	Exon/ Intron	Allele 2 cDNA	Type	Protein	Reference
RDEB-O	4	p.Gly150Arg	c.448G>A	IVS5	c.682+1G>A	SS	predicted retention of intron 5 + p.Pro228fsX32	(Drera *et al.*, 2009)
RDEB-sev gen	4	**p.Gly174Arg**	c.520G>A	4	c.520G>A	GS[a]	p.Gly174Arg	**This study**
RDEB-O	32	p.Gly1314Arg	c.3940G>A	78	c.6422delG	PTC	p.G2141EfsX64	(Kern *et al.*, 2009a)
RDEB	33	p.Gly1332Asp	c.3995G>A	ND	ND	ND	ND	(Garcia *et al.*, 2009)
RDEB-O	34	p.Gly1338Arg	c.4012G>A	IVS34	c.4048-1G>A	SS	Predicted skipping of exon 35 (in-frame; 24aa)	(van den Akker *et al.*, 2009)
RDEB-sev gen	34	p.Gly1338Val	c.4013G>T[b]	ND	ND	ND	ND	(Dang *et al.*, 2007)
RDEB-O	34	p.Gly1347Arg	c.4039G>C	70	c.5820G>A	SS	Predicted skipping of exon 70 (in-frame; 16aa)	(Terracina *et al.*, 1998)
RDEB-O	34	p.Gly1347Trp	c.4039G>T	IVS30	c.3832-2A>G	SS	in-frame deletion	(Kern *et al.*, 2006)
RDEB-O	36	p.Gly1383Arg	c.4147G>A	80	c.6527insC	PTC	p.Pro2176fsX113	(Escamez *et al.*, 2010)
RDEB-O	42	**p.Gly1483Asp**	c.4448G>A	42	c.4448G>A	GS	p.Gly1483Asp	**This study**
RDEB-O	42	p.Gly1492Arg	c.4474G>A	4	c.497insA	PTC	p.Gln166fsX51	(Kern *et al.*, 2006)

RDEB-BDN	44	p.Gly1519Asp	c.4556G>A	86	c.6752G>A	GS	p.Gly2251Glu	(Hammami-Hauasli *et al.*, 1998a)
RDEB-O	45	p.Gly1522Arg	c.4564G>C	ND	ND	ND	ND	(Kern *et al.*, 2006)
RDEB-pr	48	**p.Gly1572Ala**	c.4715G>C	104	c.7786delG	PTC	p.Gly2596fsX49	**This study**
RDEB-O	50	p.Gly1595Arg	c.4783G>C	115	c.8479C>T	PTC	p.Gln2827Stop	(Sato-Matsumura *et al.*, 2002)
RDEB-O	50	p.Gly1604Arg	c.4810G>A	72	c.5964insC	PTC	p.Gly1988fsX110	(Whittock *et al.*, 1999)
RDEB-O	51	p.Gly1616Arg	c.4846G>A	51	c.4846G>A	GS	p.Gly1616Arg	(Kern *et al.*, 2006)
RDEB-O	53	p.Gly1652Arg	c.4954G>A	3	c.425A>G	SS	p.Lys142Arg	(Cserhalmi-Friedman *et al.*, 1997)
RDEB-O	53	p.Gly1655Gly	c.4965C>T[b]	74	c.6187C>T	MS	p.Arg2063Trp	(Gardella *et al.*, 2002)
RDEB-O	54	p.Gly1664Ala	c.4991G>C	54	c.4991G>C or c.497insA or c.425A>G	GS PTC SS[c]	p.Gly1664Ala p.Gln166fsX51 p.Lys142Arg	(Gardella *et al.*, 2002)
RDEB-O	54	p.Gly1673Arg	c.5017G>A	ND	ND	ND	ND	(Dang *et al.*, 2007)
RDEB-O	56	p.Gly1696Glu[d]	c.5090G>A	84	c.6691insC	PTC	p.Pro2231fsX58	(Salas-Alanis *et al.*, 2000)
RDEB-O	56	p.Gly1703Glu	c.5108G>A	69	c.5772delG	PTC	p.Glu1924fsX50	(Whittock *et al.*, 1999)
RDEB-O	58	p.Gly1719Arg	c.5155G>C	58	c.5155G>C	GS	p.Gly1719Arg	(Kern *et al.*, 2006)
RDEB-sev gen	61	**p.Gly1770Ser**	c.5308G>A	61	c.5308G>A	GS[e]	p.Gly1770Ser	**This study**
RDEB-O	61	p.Gly1782Arg	c.5344G>A	73	c.6081delC	PTC	p.Pro2027fsX178	(Christiano *et al.*, 1996a)
RDEB	61	p.Gly1782Val	c.5345G>T	3	c.425A>G	SS[c]	p.Lys142Arg	(Kern *et al.*, 2006)

RDEB-O	63	p.Gly1812Arg	c.5434G>C	31	c.3857delA	PTC	p.Gln1286fsX38	(Masunaga *et al.*, 2000)
RDEB-O	63	p.Gly1815Arg	c.5443G>A	70	c.5818delC	PTC	p.Pro1940fsX64	(Sato-Matsumura *et al.*, 2002)
RDEB-O	65	p.Gly1845Arg	c.5533G>A	3	c.425A>G	SS[c]	p.Lys142Arg	(Jerabkova *et al.*, 2010)
RDEB-sev gen	66	p.Gly1857Glu	c.5570G>A	66	c.5570G>A	GS	p.Gly1857Glu	(Kern *et al.*, 2009a)
RDEB-i	68	p.Gly1907Glu	c.5720G>A	105	c.7805G>A	GS	p.Gly2602Glu	(van den Akker *et al.*, 2009)
RDEB-i	68	**p.Gly1907Asp**	c.5720-21 GA>AT	54	c.5047C>T	PTC	p.Arg1683Stop	**This study**
RDEB-O	68	p.Gly1907Asp[f]	c.5723G>A	13 76	c.1732C>T or c.6311del2	PTC PTC	p.Arg578Stop p.Ser2104fsX11	(Varki *et al.*, 2007)
RDEB-BDN	68	p.Gly1910Ser	c.5728G>A	IVS5	c.682+1G>A	SS	predicted retention of intron 5 + p.Pro228fsX32	(Hashikawa *et al.*, 2009)
RDEB-sev gen	72	p.Gly1982Trp	c.5944G>T	5	c.553C>T	PTC	p.Arg185Stop	(Hovnanian *et al.*, 1997)
RDEB-O	72	**p.Gly1982Glu**	c.5945G>A	12	c.1573C>T	PTC	p.Arg525Stop	**This study**
RDEB-O	73	p.Gly2009Arg	c.6025G>A	115	c.8523del14	SS	predicted skipping of exon 115 (in-frame; 29aa)	(Winberg *et al.*, 1997)
RDEB	73	p.Gly2025Ser	c.6073G>A	ND	ND	ND	ND	(Pfendner *et al.*, 2003)

RDEB-O	73	p.Gly2025Ala	c.6074G>C	105	c.7828C>A	PTC	p.Arg2610Stop	(Hovnanian *et al.*, 1997)
RDEB-O	73	Gly2028Trp	6082G>T	117	8698del11	PTC	p.Ser2900fsX19	(Varki *et al.*, 2007)
RDEB- sev gen	73	p.Gly2028Arg	c.6082G>A	13	c.1661del57	PTC	NR	(Varki *et al.*, 2007)
RDEB- sev gen	73	p.Gly2031Ser	c.6080G>A[g]	73	c.6080G>A	GS	p.Gly2031Ser	(Nordal *et al.*, 2001)
RDEB-sev gen	73	p.Gly2049Glu	c.6146G>A	34	c.4027C>T	PTC	p.Arg1343Stop	(Hovnanian *et al.*, 1997)
RDEB-O	73	p.Gly2058Ala	c.6173G>C	102	c.7621C>T	PTC	p.Arg2541Stop	(Kern *et al.*, 2009a)
RDEB-sev gen	74	p.Gly2061Glu	c.6182G>A	IVS116	c.8620+26G>A	SS	ND	(Chao and Lee, 2007)
RDEB-O	75	p.Gly2073Asp*	c.6218G>A	13	c.1732C>T	PTC	p.Arg578Stop	(Dunnill *et al.*, 1996)
RDEB-i	75	p.Gly2088Arg	c.6262 G>A	5	c.676 C>T	PTC	p.Arg226Stop	(Chiaverini *et al.*, 2010)
RDEB-O	75	p.Gly2088Glu	c.6263G>A	116	c.8569G>T	PTC	p.Glu2857Stop	(Suzuki *et al.*, 2006)
RDEB-O	78	p.Gly2132Ser	c.6394G>A	IVS5	c.682+1G>A	SS	predicted retention of intron 5 + p.Pro228fsX32	(Kern *et al.*, 2009a)
RDEB-O	78	p.Gly2132Asp	c.6395G>A	58	c.5188C>T	PTC	p.Arg1730Stop	(Whittock *et al.*, 1999)
RDEB-O	82	p.Gly2192Ser	c.6574G>A	73	c.6081delC	PTC	p.Pro2027fsX178	(Whittock *et al.*, 1999)
RDEB-sev gen	82	p.Gly2204Ser	c.6610G>A	55	c.5066C>G	PTC	p.Ser1689XStop	(Kim *et al.*, 2003)

RDEB-sev gen	83	p.Gly2210Val	c.6629G>T	113	c.8371C>T	MS	p.Arg2791Trp	(Varki *et al.*, 2007)
RDEB-O	83	**p.Gly2213Arg**	c.6637G>A	52	c.4918delG	PTC	p.Gly1640fsX69	**This study**
RDEB-O	84	**p.Gly2221Arg**	c.6661G>A	49	c.4748delG	PTC	p.Gly1583fsX126	**This study**
RDEB-O	84	p.Gly2221Ala	c.6662G>C	IVS106	c.7930-1G>C	SS	predicted skipping of exon 106 (in-frame, 18aa)	(Escamez *et al.*, 2010)
RDEB-O	84	p.Gly2233Cys	c.6697G>T	84	c.6695C>G	MS	p.Pro2232Arg	(Ryoo *et al.*, 2001)
RDEB-O	84	p.Gly2233Ser	c.6697G>A	ND	ND	ND	ND	(Salas-Alanis *et al.*, 2000)
RDEB- sev gen	86	p.Gly2251Ala/ p.P1458L	c.6752G>C	103	c.7723G>A	GS	p.Gly2575Arg	(Varki *et al.*, 2007)
RDEB-BDN	86	p.Gly2251Glu	c.6752G>A	44	c.4556G>A	GS	p.Gly1519Asp	(Hammami-Hauasli *et al.*, 1998a)
RDEB-O	86	p.Gly2263Val	c.6788G>T	13	c.1732C>T	PTC	p.Arg578Stop	(Whittock *et al.*, 1999)
RDEB-O	86	**p.Gly2272Ala**	c.6815G>C	21	ND	ND	ND	**This study**
RDEB-O	87	p.Gly2287Arg	c.6859G>A	89	c.6946G>A	GS	p.Gly2316Arg	(Shimizu *et al.*, 1999)
RDEB-O	87	p.Gly2296Glu	c.6887G>A	12	c.1573C>T	PTC	p.Arg525Stop	(Jerabkova *et al.*, 2010)
RDEB-O	89	p.Gly2316Arg	c.6946G>A	87	c.6859G>A	GS	p.Gly2287Arg	(Shimizu *et al.*, 1999)
RDEB-sev gen	91	p.Gly2351Arg	c.7051G>A	56	c.5103delCCinsG	PTC	p.Gly1701fsX8	(Christiano *et al.*, 1996a)
RDEB-O	92	p.Gly2357Ser	c.7069G>A	ND	ND	ND	ND	(Varki *et al.*, 2007)
RDEB-O	92	**p.Gly2357Arg**	c.7069G>C	IVS64	c.5487+4delAGTG	PTC	in-frame deletion	**This study**

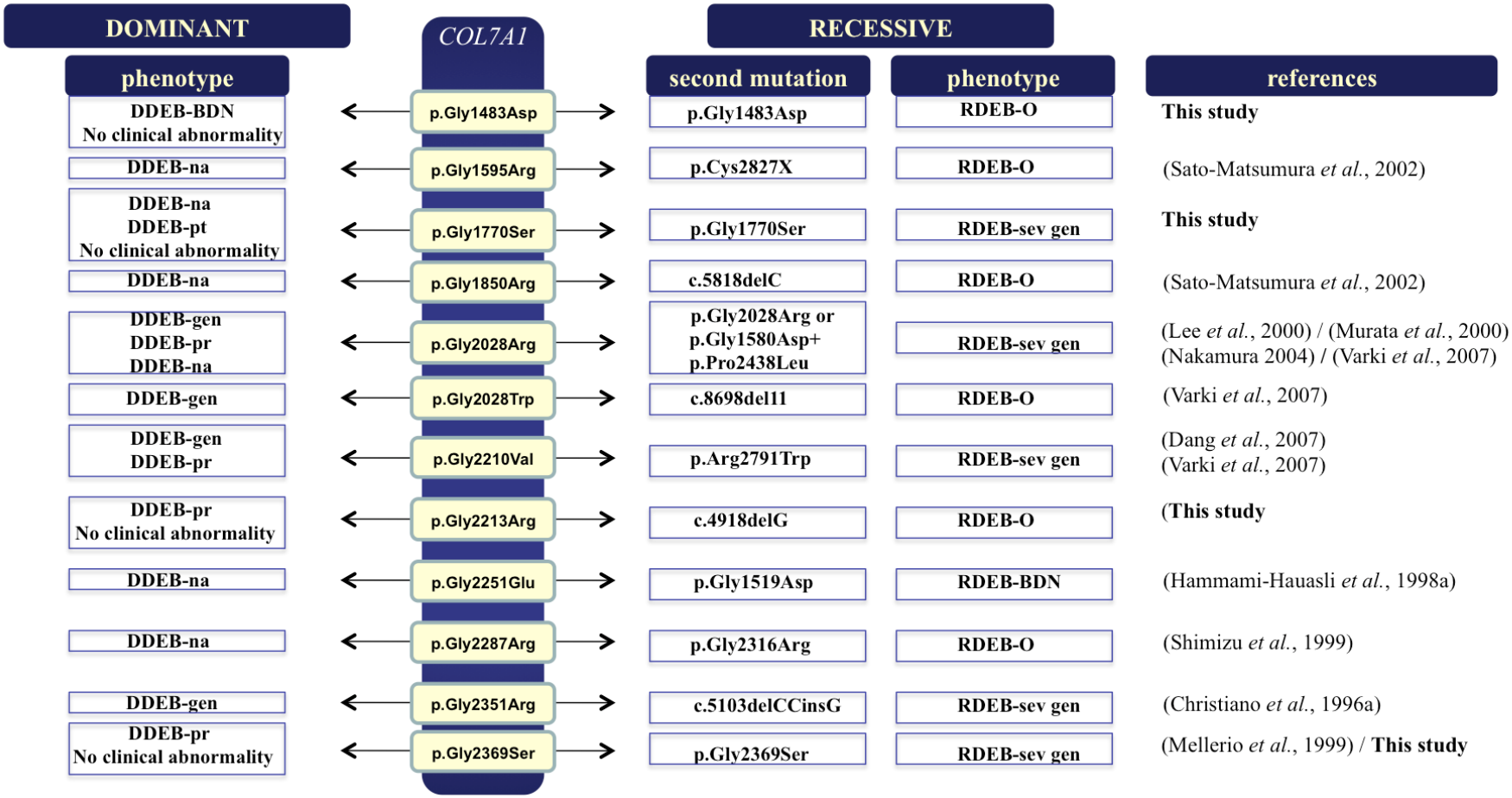
RDEB-O	92	p.Gly2366Cys	c.7096G>T	70	c.5818delC	PTC	p.Pro1940fsX64	(Sawamura *et al.*, 2005)
RDEB-O	92	p.Gly2366Ser	c.7096G>A	74	c.6187C>T	MS	p.Arg2063Trp	(Hashimoto *et al.*, 1999)
RDEB-sev gen	92	p.Gly2366Asp	c.7097G>A	80	c.6527insC	PTC	p.Prol2176fsX113	(Escamez *et al.*, 2010)
RDEB-sev gen	93	**p.Gly2369Ser**	c.7105G>A	93	c.7105G>A	GS	p.Gly2369Ser	**This study**
RDEB-BDN	93	**p.Gly2372Val**	c.7115G>T	9	c.7115G>T	GS	p.Gly2372Val	**This study**
RDEB-sev gen	93	p.Gly2375Ser[h]	NR	55	c.5097G>A	SS	in-frame deletion	(Fassihi *et al.*, 2006)
RDEB-sev gen	94	p.Gly2395Asp	c.7184G>A	2	c.154delG	del	in frame deletion	(Mayama *et al.*, 1999)
RDEB-O	94	p.Gly2413Arg	c.7237G>A	20	c.2699G>A	PTC	p.Trp900Stop	(van den Akker *et al.*, 2009)
RDEB	94	p.Gly2413Glu	7238G>A	3	c.425A>G	SS[c]	p.Lys142Arg	(Kern *et al.*, 2006)
RDEB-O	94	p.Gly2422Val	7265G>T	94	c.7265G>T	GS	p.Gly2422Val	(Kraemer *et al.*, 2006)
RDEB-O	95	p.Gly2434Arg	7300G>A	80	c.6527insC	PTC	p.Prol2176fsX113	(Escamez *et al.*, 2010)
RDEB-sev gen	96	p.Gly2449Arg	c.7345G>A	96	c.7345G>A	GS	p.Gly2449Arg	(Kern *et al.*, 2009a)
RDEB-i	97	p.Gly2472Asp	c.7415G>A	14	c.1874del2	PTC	p.Ser625fsX4	(Chiaverini *et al.*, 2010)
RDEB-pt	101	p.Gly2520Val	c.7559G>T	110	c.8209G>C	GS	p.Gly2737Arg	(Escamez *et al.*, 2010)
RDEB-O	101	p.Gly2533Asp	c.7598G>A	26	c.3474delA	PTC	p.Pro1158fsX2	(Kern *et al.*, 2009a)

RDEB-sev gen	102	p.Gly2557Arg	c.7669G>A	34	c.4027C>T	PTC	p.Arg1343Stop	(Jerabkova *et al.*, 2010)
RDEB-O	103	p.Gly2569Arg	c.7705G>C	103	c.7705G>C	GS	p.Gly2569Arg	(Christiano *et al.*, 1996a)
RDEB-O	103	p.Gly2569Ser	c.7705G>A	6	c.846+1G>A	SS	predicted skipping of exon 6 (in-frame; 55aa)	(Kern *et al.*, 2009a)
RDEB-O	103	p.Gly2576Arg[i]	c.7723G>A	116	c.8569G>T	PTC	p.Glu2857Stop	(Shimizu *et al.*, 1996)
RDEB-sev gen	104	p.Gly2587Asp	c.7760G>A	80	c.6527insC	PTC	p.Prol2176fsX113	(Escamez *et al.*, 2010)
RDEB-O	104	p.Gly2590Asp	c.7769G>A	33	c.4011G>A	SS	p.Pro1337Pro	(van den Akker *et al.*, 2009)
RDEB-i	105	p.Gly2602Glu	c.7805G>A	68	c.5720G>A	GS	p.Gly1907Glu	(van den Akker *et al.*, 2009)
RDEB-O	105	**p.Gly2620Val**	c.7859G>T	105	c.7864C>T	MS	p.Arg2622Trp	**This study**
RDEB-O	105	p.Gly2623Ser	c.7867G>A	70	c.5818delC	PTC	p.Pro1940fsX64	(Sawamura *et al.*, 2006a)
RDEB-O	107	p.Gly2653Arg	c.7957G>A	97	c.7411C>T	PTC	p.Arg2471Stop	(Christiano *et al.*, 1996a)
RDEB-pr	107	**p.Gly2656Val**	c.7967G>T	65	c.5564ins28	PTC	p.Arg1855fsX34	**This study**
RDEB-sev gen	108	p.Gly2671Val	c.8012G>T	108	c.8012G>T	GS	p.Gly2671Val	(Kon *et al.*, 1997a)
RDEB-sev gen	108	p.Gly2674Arg	c.8020G>C	79	c.6501G>A	SS	in-frame deletion	(Christiano *et al.*, 1996a)
RDEB-O	108	p.Gly2674Asp	c.8021G>A	13	c.1732C>T	PTC	p.Arg578Stop	(Whittock *et al.*, 1999)
RDEB-O	109	p.Gly2689Arg	c.8065G>A	80	c.6527insC	PTC	p.Prol2176fsX113	(Kern *et al.*, 2006)
RDEB	109	p.Gly2695Ser	c.8083G>A	109	c.8083G>A	GS	p.Gly2695Ser	(Varki *et al.*, 2007)

RDEB-sev gen	110	p.Gly2719Ala	c.8156G>C	ND	ND	ND	ND	(Fassihi *et al.*, 2006)
RDEB	110	p.Gly2737Arg	c.8209G>C	ND	ND	ND	ND	(Kern *et al.*, 2006)
RDEB-sev gen	110	p.Gly2740Ala	c.8219G>C	104	c.7786delG	PTC	p.Gly2596fsX49	(Whittock *et al.*, 1999)
RDEB-sev gen	111	p.Gly2749Arg	c.8245G>A	111	c.8245G>A	GS	p.Gly2749Arg	(Christiano *et al.*, 1996a)
RDEB-O	112	p.Gly2775Ser	c.8323G>A	3	c.425A>G	SS[c]	p.Lys142Arg	(Kon *et al.*, 1998)
RDEB-BDN	112	**p.Gly2781Arg**	c.8341G>A	51	c.4894C>T	PTC	p.Arg1632Stop	**This study**

RDEB: recessive dystrophic epidermolysis bullosa; RDEB-sev gen: severe generalized; RDEB-O: other; RDEB-BDN: bullous dermolysis of the newborn; RDEB-na: nail dystrophy; RDEB-pr: pruriginosa; RDEB-i: inversa; GS: glycine substitution; IVS, intervening sequence (intron); MS: missense; N: normal; PTC: premature termination codon; ND: not determined; NR: not recorded; SS: splice site; bold: new mutations. * de novo mutations. [a]within donor splice site consensus sequence, [b]results in a premature termination codon; [c]results in the loss of a StyI restriction site with various mRNA isoforms (Gardella et al. Am J Hum Genet 1996; 59: 292-300); [e]within acceptor splice site consensus sequence; corrections: [d]p.Gly1696Glu, [f]p.Gly1907Asp, [g]c.6080G>A, [h]p.Gly2375Ser, [i]p.Gly2576Arg but corrected nomenclature should be: p.Gly1697Glu, p.Gly1908Asp, c.6091G>A, p.Gly2377Ser, p.Gly2575Arg respectively.

Figure 4.2 GS mutations with both dominant and recessive inheritance patterns.



4.3.1 The phenotype expression variability of the mutation p.Gly1483Asp

The p.Gly1483Asp mutation (c.4448G>A) leads to BDN, mild localised DDEB or no phenotype when inherited on one allele; but results in mild RDEB when inherited on both alleles as detailed below (Figure 4.3).

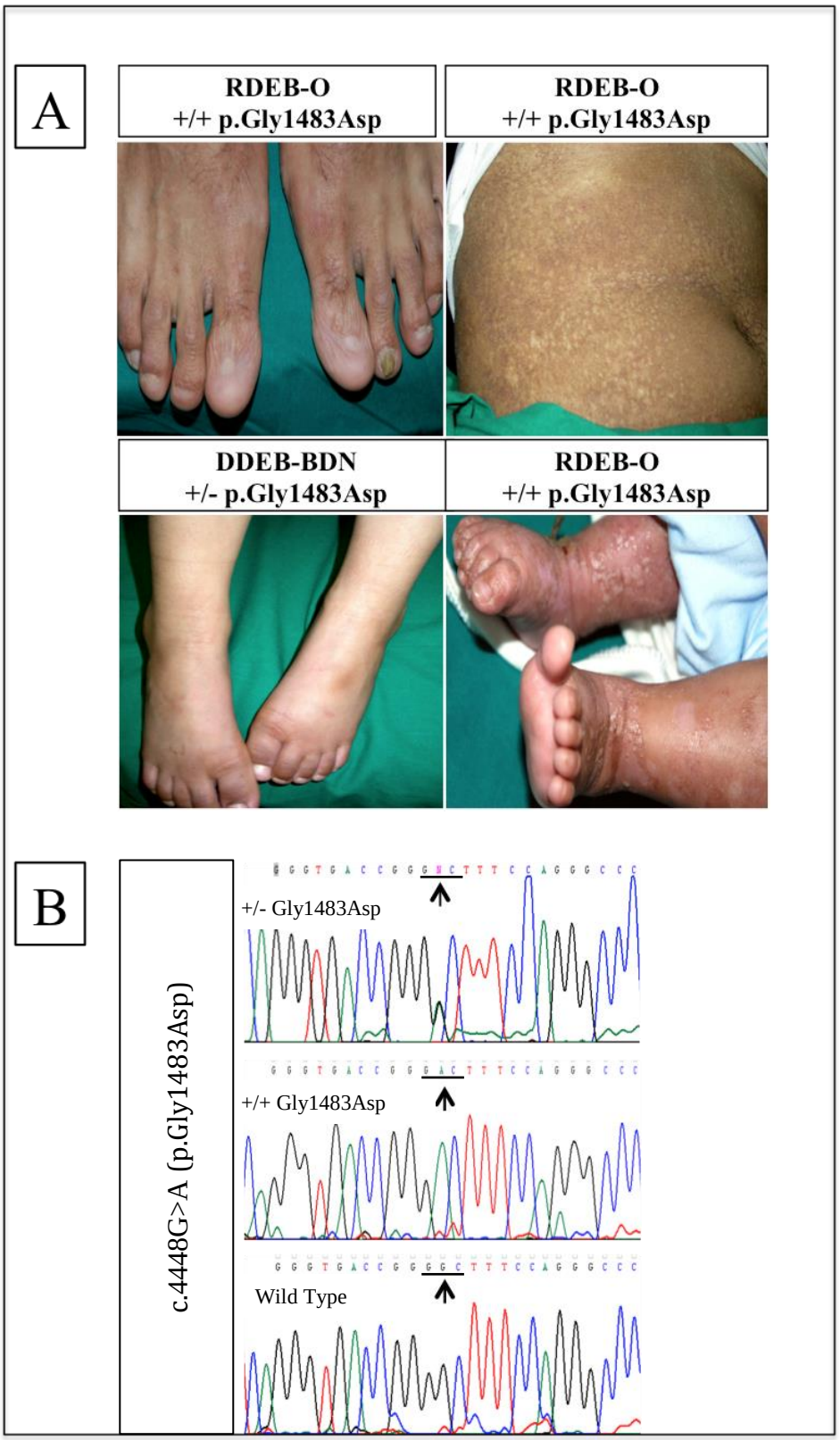
BDN was diagnosed in a 7-year-old Kuwaiti female whose parents were first cousins. She developed generalised blistering at birth, which healed with minimal scarring and a few milia. However, the frequency of blistering decreased with age and had completely ceased by the age of 4 months suggestive of a diagnosis of BDN. She was found to be heterozygous for the GS mutation p.Gly1483Asp.

A homozygous p.Gly1483Asp mutation was also identified in a 16-month old Kuwaiti male. His parents were distant cousins and there was no known relationship to the previous case. Fragility of the skin was noticed soon after birth with spontaneous blistering of the hands, trunk and extremities, which healed with minimal scarring and milia formation. This was associated with occasional oral erosions. The frequency of blistering decreased with advancing age. Collectively, the phenotype was suggestive of mild RDEB and mutation analysis confirmed the presence of a homozygous mutation p.Gly1483Asp. Neither of his parents, heterozygous for p.Gly1483Asp, had any clinical abnormalities.

Furthermore, a 17-year-old male and his 23-year-old sister of Kuwaiti origin, with no relation to the previous 2 cases, were diagnosed with RDEB-O. Their parents were first cousins with double consanguinity. Both children had generalised blistering at birth with recurrent oral ulcers. The frequency of blister formation decreased with age although there was persistent skin fragility

as well as albopapuloid lesions on the chest, abdomen and upper back, n addition to mild inflammation of the gums. There was nail dystrophy with loss of several finger and toe nails suggestive of RDEB-O. Both individuals were homozygous for the mutation p.Gly1483Asp. Their parents were heterozygous carriers of this mutation and were clinically unaffected.

Figure 4.3 The GS mutation p.Gly1483Asp leads to both dominant and recessive DEB. (A) p.Gly1483Asp results in heterogenous phenotypes; BDN and RDEB-O. (B) DNA sequencing shows heterozygosity or homozygosity for the mutation p.Gly1483Asp.



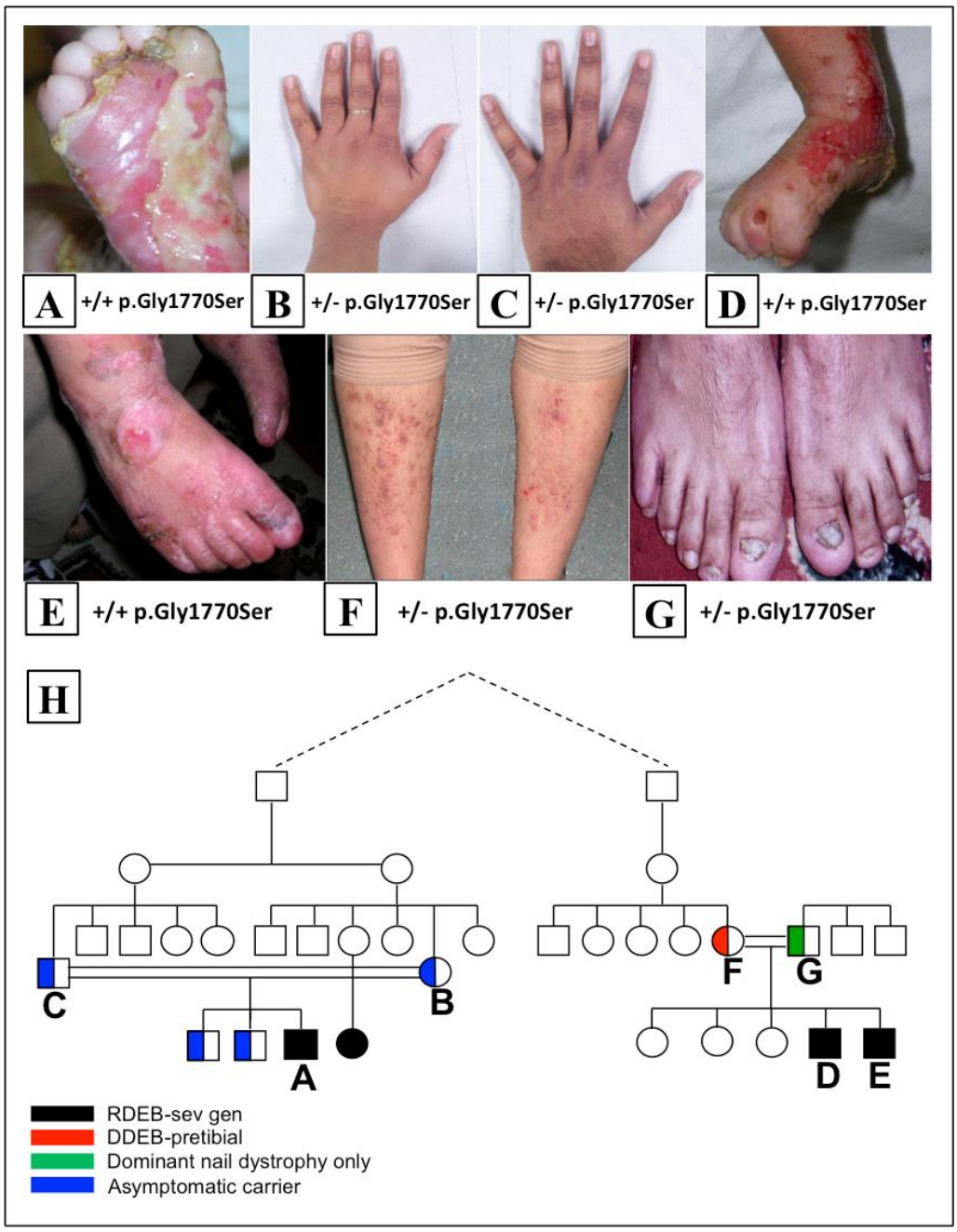
4.3.2 The phenotype expression variability of the mutation p.Gly1770Ser

Heterozygosity for the mutation p.Gly1770Ser results in no phenotype, mild nail dystrophy or pretibial DDEB whereas homozygosity results in severe generalised RDEB as detailed below (Figure 4.4).

The proband is a 9-month-old Pakistani male who is related to two affected brothers aged 1 year and 4 years. All three individuals presented with skin fragility at birth. They all subsequently progressed to develop typical features of RDEB-sev gen. Mutational analysis revealed homozygosity for the glycine substitution p.Gly1770Ser, consistent with this being a recessive GS. The proband's parents were both heterozygous carriers with no skin or nail pathology. Thus, in both parents the GS p.Gly1770Ser appears to function as a recessive allele, which is silent in the heterozygous state.

However, the brothers' father was found to heterozygous for p.Gly1770Ser but had evidence of nail dystrophy without skin blistering. Their mother who is also heterozygous for p.Gly1770Ser had features of pretibial DEB and nail dystrophy demonstrating effects of a dominant GS.

Figure 4.4 The glycine mutation p.Gly1770Ser leads to both dominant and recessive disease in two distantly related Pakistani pedigrees. (A-G) Clinical illustration of family members showing varying degrees of skin fragility as well as a normal phenotype. (H) Pedigree shows the presence/ absence of dominant or recessive disease in relation to the genotype. RDEB-sev gen; recessive dystrophic epidermolysis bullosa-severe generalised, DDEB; dominant dystrophic epidermolysis bullosa.

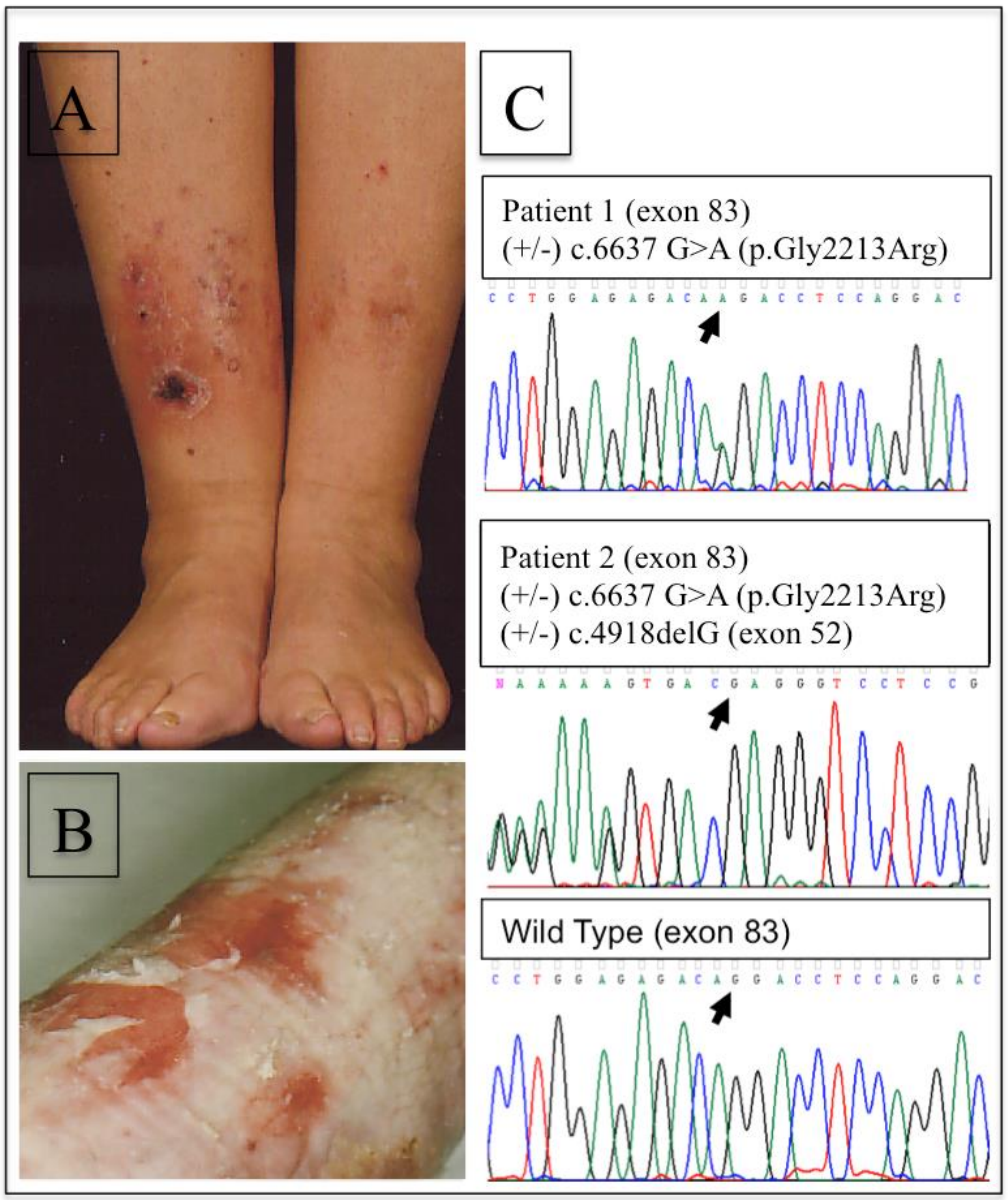


4.3.3 The phenotype expression variability of the mutation p.Gly2213Arg

The homozygous p.Gly2213Arg mutation leads to no phenotype or dominant EB-pr; but when inherited *in trans* with a frameshift mutation results in RDEB-O as detailed below (Figure 4.5).

EB-pr was diagnosed in a 45-year-old British female with blistering and itching affecting the lower legs since the age of 37 years. Clinically, there was evidence of prurigo-like lesions on her shins and she was found to be heterozygous for the GS mutation p.Gly2213Arg, supporting the diagnosis of dominant EB-pr. On the other hand, a 24-year-old British male had generalised blistering affecting the skin, eyes and oral mucosa. There was also evidence of scarring consistent with RDEB-O. He was found to be compound heterozygote for the mutation p.Gly2213Arg and the frameshift mutation c.4918delG in exon 52. Neither parent, including the carrier of the GS p.Gly2213Arg, had any clinical abnormalities.

Figure 4.5 The glycine substitution mutation p.Gly2213Arg leads to both dominant and recessive disease. (A) A 45-year-old female with dominant EB pruriginosa. (B) A 24-year-old male with clinical features of mild RDEB on the lower leg. (C) DNA sequencing of both cases compared to wild type.



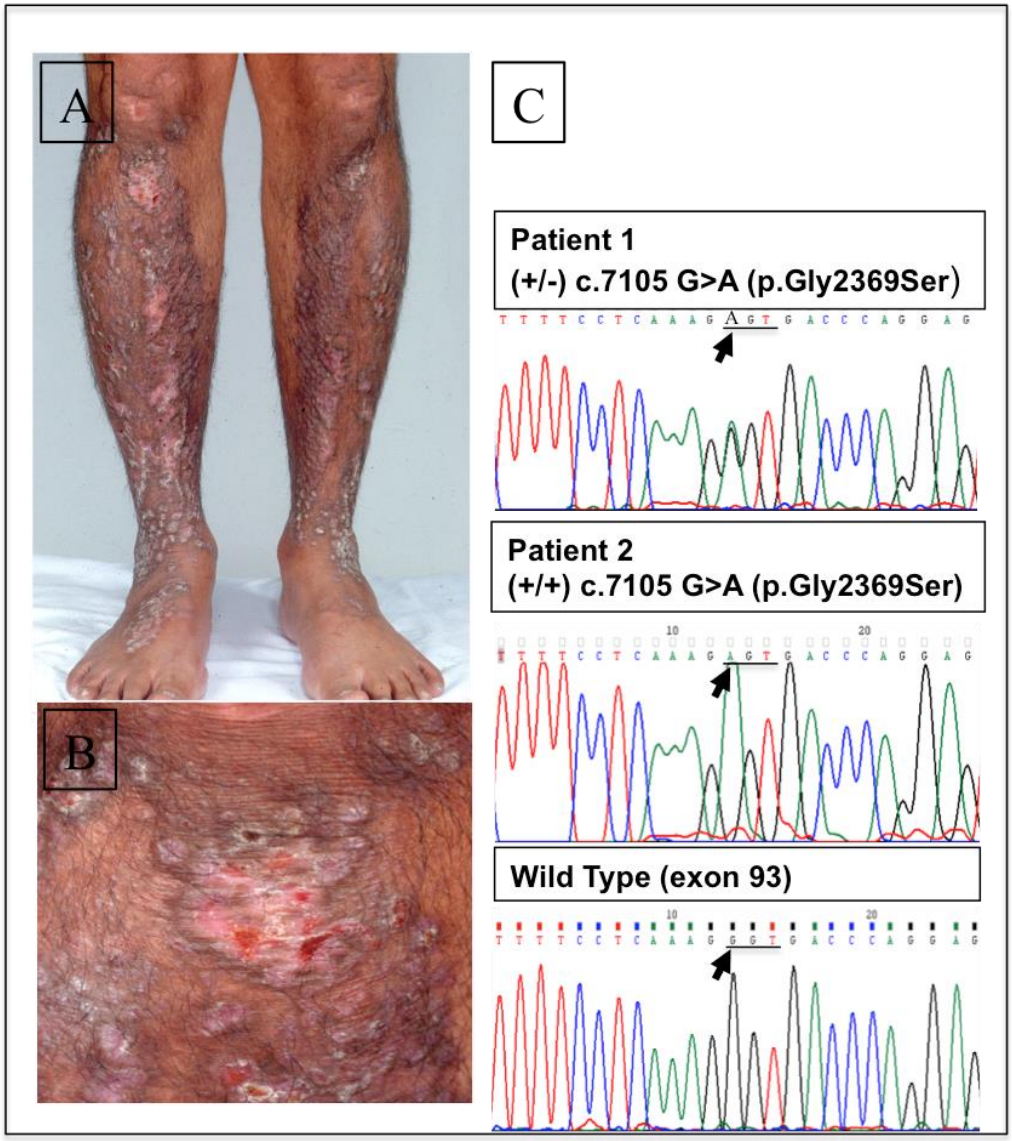
4.3.4 The phenotype expression variability of the mutation p.Gly2369Ser

The heterozygous mutation p.Gly2369Ser leads to no phenotype or dominant EB-pr whereas homozygosity results in RDEB-O as detailed below (Figure 4.6).

EB-pr was diagnosed in a 49-year-old male of Pakistani origin who had involvement of the forearms, shins, hands, feet and nails. Onset of EB was recorded at 6 months. There were also extensive lichenified prurigo-like lesions on the shins associated with itching consistent with EB-pr. Although the intense pruritus and a relative widespread involvement favoured the diagnosis of EB-pr, the features were also similar to those found in patients with pretibial EB, in whom a clear clinical distinction between these two overlapping clinical entities is sometimes difficult.

A heterozygous GS mutation; p.Gly2369Ser indicated dominantly inherited disease. An unrelated 6-year-old female had skin fragility mainly affecting the ankles and knees with occasional oral involvement. There was also evidence of progressive flexion contractures and webbing of her fingers suggestive of RDEB-sev gen. She was found to be homozygous for the GS mutation p.Gly2369Ser. Her parents were both heterozygous carriers of this mutation with no clinical abnormality.

Figure 4.6 The glycine substitution mutation p.Gly2369Ser leads to dominant EB pruriginosa or severe generalised RDEB. (A and B) A 49-year-old Pakistani male with features of EB-pr on the shins. (C) DNA sequencing of both cases compared to wild type. EB-pr; epidermolysis bullosa pruriginosa.



4.4 Discussion

This study revealed that the spectrum of GS substitution mutations is quite variable with resultant phenotypic heterogeneity encompassing all DEB subtypes. Factors affecting this heterogeneous expression could include the nature of the substituted amino acid, the position of the GS in the triple helix, the proximity of the GS to the non-helical hinge region in the triple helix or the non-collagenous NC1 or NC2 domains, unpredicted consequences of the nucleotide substitution on splicing, as well as biochemical changes to the collagen affecting helix formation, protein folding, thermal stability, intracellular transport, secretion, and assembly into anti-parallel dimers or anchoring fibrils (Shimizu *et al.*, 1996; Christiano *et al.*, 1996a; Hammami-Hauasli *et al.*, 1998b; Lee *et al.*, 2000; Sato-Matsumura *et al.*, 2002; Kern *et al.*, 2006; Chiaverini *et al.*, 2010; Chen *et al.*, 2001; Chen *et al.*, 2002a; Brittingham *et al.*, 2005; Fritsch *et al.*, 2009; Hyde *et al.*, 2006).

4.4.1 Effects of the type of GS mutations

Whilst arginine was found to be the most common amino acid (AA) to substitute glycine, there is a wide spectrum of GS each resulting in one or more DEB subtypes. This is supportive of a previous study by Persikov *et al.*, in which the predicted type of GS mutations in *COL1A1, COL1A2, COL3A1, COL4A5, and COL7A1* -as calculated by using single–base pair substitution rates- were compared with the observed GS leading to disease. *COL7A1* was unique in that no significant difference was noted between the spectrum of GS predicted and observed in the α1(VII) chains suggesting that although arginine was the most common substitute, any GS in *COL7A1* regardless of the type

will lead to pathology (Persikov *et al.*, 2004). This is also a reflection of the high evolutionary conservation of the *COL7A1* structure (Kivirikko *et al.*, 1996). In some cases glycine can be substituted by different amino acids in the same codon resulting in heterogeneous phenotypes. This is particularly evident for some GS mutation in exon 73, a frequent region for *COL7A1* mutations. The substitution of glycine in position p.Gly2028 by arginine can lead to either DDEB-gen or DDEB-pr, whereas a substitution by tryptophan, alanine or glutamic acid results in DDEB-gen (Lee *et al.*, 2000; Murata *et al.*, 2000; Varki *et al.*, 2007). In other cases different amino acid substitutions in the same codon had no effect on phenotype. For example, substitution of glycine in position p.Gly2242 by either tryptophan, glutamic acid or arginine results in EB-pr (Lee *et al.*, 1997; Tamai *et al.*, 1998; Shi and Feng, 2009).

Table 4.3 The types of amino acids substituting glycine in *COL7A1* GS mutations.

Glycine Substitution Mutations			
Amino Acid	**Symbol**	**DDEB n (%)**	**RDEB n (%)**
Arginine	Arg	29 (29.8)	39 (36.7)
Aspartic Acid	Asp	18 (18.5)	14 (13.2)
Glutamic Acid	Glu	20 (20.6)	12(11.3)
Valine	Val	12 (12.3)	10 (9.4)
Cysteine	Cys	1 (1.0)	2 (1.8)
Serine	Ser	5 (5.1)	16 (15)
Tryptophan	Trp	6 (6.1)	3 (2.8)
Alanine	Ala	6 (6.1)	9 (8.4)
Glycine	Gly	0 (0)	1 (0.9)
	Total	97	106

4.4.2 Effects of the location of GS mutations

Some studies have suggested that the location of GS could explain the level of protein expression, degree of triple helical instability and subsequent disease severity. The NC2 domain and the adjacent collagenous triple helix were found to initiate the assembly of collagen VII triple helices and mediate the formation of anti-parallel dimers (Chen *et al.*, 2001). Some GS mutations in or near this region (p.Gly2749Arg, p.Gly2569Arg, p.Gly2575Arg or p.Gly2623Cys) (Chen *et al.*, 2002a; Woodley *et al.*, 2008; Brittingham *et al.*, 2005) were found to impair collagen VII triple helix assembly resulting in RDEB. However, the novel GS p.Gly2781Arg mutation identified in this study is in closest proximity to the NC2 domain and even when inherited *in trans* with the nonsense mutation p.Arg1632Stop leads only to bullous dermolysis of the newborn, the mildest form of RDEB. Furthermore, some GS mutations in close proximity to the 'hinge region' are thought to interfere with protein folding (p.Gly2006Asp, p.Gly2034Arg, p.Gly2015Glu and p.Arg2008Glu) leading to intracellular retention of collagen VII (Chen *et al.*, 2002a; Hammami-Hauasli *et al.*, 1998a). However, according to the collective data from this study, 43% of dominant GS mutations occur in exons 73-75 which are adjacent to the hinge area, and display gross phenotypic variability that would be difficult to explain based on intracellular retention alone.

The effect of GS location in relation to renucleation sequences necessary for triple helix formation has also been studied. Nucleation refers to an early process in DNA helix formation in which 2 single stranded segments of DNA are brought in close proximity prior to propagation. The type and location of renucleation sequences at which this process occurs, in relation to

missense mutations can affect helical stability and DNA segment elongation, with subsequent effects on protein expression (Hyde *et al.*, 2006). Using peptide models, the presence of hydroxyproline rich renucleation sequences in the collagen I gene resulted in the formation of stable triple helices, whereas their absence led to unfolding of the helix N-terminal causing severe osteogenesis imperfecta. It was postulated that the distance between mutations and the next renucleation sequence might predict the level of folding impairment and hence clinical severity (Hyde *et al.*, 2006).

In addition, some recurrent mutations or 'hotspots' have been identified in specific populations such as the Arg578Stop, 7786delG and Arg2814Stop mutations in British families (Mellerio *et al.*, 1997; Mohammedi *et al.*, 1999); 2470insG in Mexican families (Salas-Alanis *et al.*, 2000; Salas-Alanis and McGrath, 2006); 5818delC, 6573+1G→C and E2857Stop in Japanese families (Tamai *et al.*, 1999), Gly1664Ala in Italian families (Gardella *et al.*, 2002), as well as the Gly2043Arg mutation in exon 73 and 425A→G worldwide (Mellerio *et al.*, 1998; Murata *et al.*, 2004). Moreover, with regards to GS mutations in particular, there appears to be a clustering of GS mutations within exon 73, however, the nature of these pathogenic mutations in both DDEB and RDEB, is similar, and to date no definite correlation has been found between the type or location of the substitution and the resulting subtype (Christiano *et al.*, 1996c).

4.4.3 Functional effects of GS mutations

As described in Chapter 1, collagen VII fibrillogeneis is a dynamic process involving the formation of pro-collagen VII then secretion to the

extracellular space where pro-collagen is processed to collagen VII homotrimers. This is followed by dimerization, polymerisation and assembly to form anchoring fibrils (Burgeson *et al.*, 1990; Burgeson, 1993; Rattenholl *et al.*, 2002; Bruckner-Tuderman *et al.*, 1995). Crucial to fibril integrity is the unique suprastructural configuration which is facilitated by interruptions to the Gly-*X*-*Y* repeats that confer flexibility necessary for collagen VII suprastructural folding (Christiano *et al.*, 1994c; Christiano *et al.*, 1994b). Finally fortification of dermal-epidermal adhesion is mediated by attachment of the anchoring fibrils to the BM via the NC1 domains (Burgeson, 1993). Mutations affecting any aspect of collagen VII fibrillogeneis will have a deleterious effect on basement membrane integrity resulting in dermal-epidermal cleavage and sub-lamina densa blisters characteristic of DEB. GS mutations can lead to secretion of a mutant peptide and incorporation into anchoring fibrils to produce dominant negative interference, as typically occurs in dominant DEB with resultant impaired stability or transport of the mutant collagen VII. Alternatively, GS mutations can result in a non-functional allele in RDEB when inherited as a homozygous mutation or *in trans* with a second loss of function mutation on the other *COL7A1* allele.

Although no accurate correlation has been found between the location or type of the GS on disease expression (Christiano *et al.*, 1996c; van den Akker *et al.*, 2009), evidence is expanding regarding the functional effects of various GS mutations on fibrillogenesis, collagen VII suprastructure and function (Hovnanian *et al.*, 1997; Hammami-Hauasli *et al.*, 1998b; Hammami-Hauasli *et al.*, 1998a; Chen *et al.*, 2002a; Woodley *et al.*, 2008; Brittingham *et al.*, 2005; Fritsch *et al.*, 2009). Substitution of the glycine residue by a bulkier

residue results in structural impairment as evidenced by impaired helix to coil formation, protein folding and secretion resulting in intracellular accumulation of collagen VII (p.Gly2006Asp, p.Gly2034Arg, p.Gly2015Glu and p.Gly2749Arg) (Hammami-Hauasli *et al.*, 1998a; Chen *et al.*, 2002a; Hovnanian *et al.*, 1997), as well as abnormal dimer formation (p.Gly2749Arg p.Gly2569Arg and p.Gly2575Arg) (Chen *et al.*, 2002a; Woodley *et al.*, 2008; Brittingham *et al.*, 2005). This is also reflected in the reduced thermal stability of mutant molecules and their increased susceptibility to limited trypsin and pepsin digestion (p.Gly2689Arg, p.Gly1776Arg, p.Gly2006Asp, p.Gly2015Glu, p.Gly2749Arg, p.Gly2006Asp, p.Gly2034Arg, p.Gly2015Glu, p.Gly2569Arg, p.Gly2575Arg and p.Gly2049Glu) (Kern *et al.*, 2006; Fritsch *et al.*, 2009; Chen *et al.*, 2002a; Hammami-Hauasli *et al.*, 1998b; Woodley *et al.*, 2008). The instability caused by some of these mutations (p.Gly1776Arg, p.Gly2006Asp or p.Gly2015Glu) was shown in one study to be rescued when wild-type collagen VII molecules were over-expressed (Fritsch *et al.*, 2009). Furthermore, the stability of the collagen VII triple helix was not only integral for precise suprastructural conformation, it was also shown to be crucial for keratinocyte motility as evidenced by the effects of the GS mutation p.Gly2049Glu in recombinant *COL7A1* mutants on triple helix assembly, fibroblast adhesion and keratinocyte migration (Woodley *et al.*, 2008).

4.4.4 Genotype-phenotypic heterogeneity

In this study, it was noted that for some GS mutations (p.Gly1483Asp, p.Gly1770Ser, p.Gly2213Arg and p.Gly2369Ser), the phenotype may be variable and there is often overlap between dominant and recessive cases, a

finding reported for some other GS mutations; p.Gly1595Arg, p.Gly1815Arg, p.Gly2028Arg, p.Gly2028Trp, p.Gly2251Glu, p.Gly2287Arg and p.Gly2351Arg (Hammami-Hauasli *et al.*, 1998b; Sato-Matsumura *et al.*, 2002; Christiano *et al.*, 1996c; Varki *et al.*, 2007; Shimizu *et al.*, 1999; Lee *et al.*, 2000) (Figure 4.2). These GS mutations that can have both dominant and recessive patterns of inheritance comprise <10% of all *COL7A1* GS.

The *de novo* GS mutation p.Gly2028Arg, was described in a Chinese patient with DDEB-gen (Lee *et al.*, 2000). It was also described in 2 pedigrees with DDEB-na and DDEB-pr (Murata *et al.*, 2000; Nakamura *et al.*, 2004). However, the same mutation inherited *in trans* with a loss-of-function mutation c.1661del57 led to RDEB-gen. It was also found to result in RDEB-O when inherited *in trans* with two maternal mutations; p.Gly1580Leu and p.Pro2438Leu (Varki *et al.*, 2007). Another GS mutation in the same codon p.Gly2028Trp results in RDEB-O when inherited *in trans* with the loss-of-function mutation c.8698del11 while the mother, a carrier of the p.Gly2028Trp shows features of DDEB-gen (Varki *et al.*, 2007). Furthermore, the mutation p.Gly2210Val was described in a patient with RDEB-gen when inherited *in trans* with the missense mutation p.Arg2791Trp but interestingly resulted in DDEB-O and DDEB-pr when both mutations were inherited on the same allele in separate cases (Varki *et al.*, 2007; Dang *et al.*, 2007). The mutation p.Gly2351Arg *in trans* with the deletion/insertion mutation c.5103delCCinsG results in RDEB-O in a monozygotic twins whereas the mother, carrier of the p.Gly2351Arg had DDEB-gen (Varki *et al.*, 2007; Christiano *et al.*, 1996c). The mutation p.Gly2287Arg in association with p.Gly2316Arg resulted in RDEB-O in a Japanese girl but 3 family members, carriers of the

p.Gly2287Arg, showed evidence of nail dystrophy only (Shimizu *et al.*, 1999). Moreover, two Japanese patients with nail dystrophy had dominantly inherited mutations p.Gly1595Arg and p.Gly1815Arg, respectively. However, each had an offspring with RDEB (Sato-Matsumura *et al.*, 2002). Heterozygous p.Gly2251Glu resulted in dominantly inherited nail dystrophy however when inherited *in trans* with p.Gly1519Asp resulted in BDN (Hammami-Hauasli *et al.*, 1998a). 4 novel GS mutations with dominant-recessive inheritance were identified in this study; p.Gly1483Asp, p.Gly1770Ser; p.Gly2213Arg and p.Gly2369Ser, which have new implications for genotype-phenotype correlation.

These findings highlight the phenotypic and genotypic variability encountered in dominant and recessive cases of DEB and demonstrates the complexity in establishing accurate paradigms for genotype-phenotype correlation in DEB, particularly when a GS mutation on one *COL7A1* allele is disclosed during diagnostic gene screening. Although, it is not surprising that GS mutations account for the vast majority of mutations in the *COL7A1* and collagen genes in general, owing to the high glycine content, the presence of both dominant and recessive GS mutations is unusual. In fact, in addition to *COL7A1*, this bimodal pattern of inheritance in collagen genes has only been described for *COL1A2* in osteogenesis imperfecta, *COL4A3/COL4A4* in Alport's and benign familial haematuria, *COL6A1/COL6A2* in Ulrich's congenital muscular dystrophy and Bethlem's myopathy and *COL11A2* in osteochondrodysplasia and autosomal dominant non-syndromic hearing loss (De Paepe *et al.*, 1997; Jimenez-Mallebrera *et al.*, 2006; Lemmink *et al.*, 1996; Longo *et al.*, 2002; McGuirt *et al.*, 1999; Mochizuki *et al.*, 1994; Nagel *et al.*,

2005; Nicholls *et al.*, 1984; Pace *et al.*, 2008; Spotila *et al.*, 1991; Spotila *et al.*, 1992; van der Loop *et al.*, 2000; Vikkula *et al.*, 1995; Badenas *et al.*, 2002).

4.4.5 Modifiers of disease expression

The phenotypic heterogeneity associated with GS mutations could, in part be explained by genetic and epigenetic modifiers including variable splicing and degradation, large genomic deletions and silent mutations as well as effects of endoplasmic reticulum stress and mutant proteins on apoptosis and gene expression (Covaciu *et al.*, 2011; Nissim-Rafinia and Kerem, 2005; Bateman *et al.*, 2009; Knaup *et al.*, 2012; Kern *et al.*, 2009a; Titeux *et al.*, 2006).

On a transcriptional level, various genetic factors modulating the level of collagen VII expression have been studied including the variable splicing of transcriptional RNA associated with splice site mutations (Nissim-Rafinia and Kerem, 2005). This results in the production of alternatively spliced isomers with subsequent variability in the level of functional proteins and associated clinical effects.

Another mechanism affecting splicing was highlighted by a recent study demonstrating that a translationally silent mutation; c.6846G>C (p.Leu2282Leu), in an exonic splicing enhancer (ESE) sequence, distant from a consensus splice site in exon 87 is capable of causing exon definition and exon skipping. These sequences are capable of selecting splicing sites in a cascade that starts with binding of ESE to members of the serine⁄arginine-rich (SR) protein family and culminating in recruitment of spliceosomes and exon

skipping (Covaciu *et al.*, 2011). Similar mutations in previously unrecognised ESE sequences could also modulate *COL7A1* expression. Similarly, undetected mutations such as large genomic deletions or other silent mutations could also contribute to variable disease expression explaining phenotypic heterogeneity (Kern *et al.*, 2009a).

The degree of skin fragility can also be influenced by epigenetic mechanisms affecting the level of protein expression in the BMZ including variable degradation of intracellularly accumulated mutant proteins as well as effects of endoplasmic reticulum stress and unfolded protein response on apoptosis and altered gene expression (Bateman *et al.*, 2009). Moreover, matrix metalloproteinase-1 (*MMP-1*), a zinc metallo-endopeptidase capable of degrading extracellular proteins, was found to be transcriptionally upregulated secondary to a single nucleotide polymorphisms (1G/2G) in the *MMP-1* gene promoter resulting in a more severe RDEB phenotype (Titeux *et al.*, 2008).

Furthermore modulation of cellular mechanical stability in response to stress could have implications on disease expression in DEB. In a recent in vitro study by Knaup *et al.*, the gene expression profiles of EBS-MD, RDEB and GABEB were analysed. All revealed up-regulation of *COL16A1* and *FN1* (fibronectin gene), in addition to accumulation of cholesterol intracellularly; factors known to confer greater resistance towards mechanical forces and stabilisation of the cell membrane respectively. In addition there was down-regulation of ribosomal protein S27A, a ubiquitin fusion protein, which could potentially result in increased degradation of mutant alleles by ubiquitin (Knaup *et al.*, 2012; Redman and Rechsteiner, 1989).

In the future, particularly with the introduction of whole genome and exome sequencing as well as data mining studies, previously unrecognised modifiers are likely to be revealed (Reverter *et al.*, 2008).

It is still not clear why some glycine mutations cause pathology when in inherited dominantly while others are silent. This is further complicated by the fact that some GS mutations are inherited in both dominant and recessive mode. Collectively, the hypothesis is that, in addition to the clear cut dominant and recessive GS mutations, there exists a new entity of mutations capable of causing disease when inherited dominantly or are silent when inherited on one allele and only causing pathology when inherited *in trans* with another *COL7A1* mutation. Therefore, it is likely that there are 3 types of glycine substitution with regards to the genotypic consequences: dominant, recessive or a mixed pattern. This has implications for genotype-phenotype correlation and accurate genetic diagnosis and counselling. When one known dominant *COL7A1* GS mutation is found it is imperative to look for another mutation particularly where the clinical appearance or mode of inheritance is in doubt.

However, despite analysis of the data outlined in this study, it is difficult to predict which particular mutations are likely to be dominant or recessive, as reinforced by the identification of the 12 GS mutations that can be either dominant or recessive. In addition, amino acid substitutions in glycines 1522, 2009, 2061, 2073, 2233, 2366, 2623 and 2719 may cause dominant or recessive disease but with different substituting residues in the different disease subtypes. There does not appear to be any specific differences in males or females with regards to phenotype. Clinical heterogeneity within a specific genotype may reflect influences such as functional polymorphisms within

genes such as the matrix metalloproteinase 1 promoter (Titeux *et al.*, 2008) but this cannot impact on whether the inheritance pattern is actually dominant or recessive.

In conclusion, although a clear phenotype-genotype correlation for GS mutations was not identified, the above data provides a useful cross-referencing tool for investigators screening for *COL7A1* GS mutations. In all cases of DEB, a comprehensive screening of the *COL7A1* even if a "possible" dominant GS mutation has been identified is recommended to ensure that recessively inherited GS mutations are not missed.

Chapter 5

Dystrophic epidermolysis bullosa: the significance of missense non-glycine substitution mutations

Abstract

Glycine amino acids are integral to the *COL7A1* triple helical stability and suprastructural conformation, while alterations in non-glycine amino acids are likely to have a less consequential effect. However, the location of non-GS missense mutations, particularly in highly conserved or critical regions, have also been found to affect helix formation, protein folding, resistance to protease degradation, intracellular transport and secretion, fibroblast adhesion, keratinocyte migration as well as assembly into anti-parallel dimers and anchoring fibrils. The type of the non-GS mutations might also predict the functional consequences and resultant phenotypic severity; however, it is more likely that a combination of factors including potential genetic and epigenetic modifiers modulate collagen VII expression and the subsequent phenotype.

In this study I report 16 novel non-GS missense *COL7A1* mutations underlying DEB and highlight associated phenotypic and genotypic heterogeneity important for establishing accurate paradigms for genotype-phenotype correlation.

5.1 Introduction

DEB results from mutations in *COL7A1* leading to qualitative and quantitative alterations of the anchoring fibrils at the DEJ with subsequent skin fragility. Both autosomal dominant and recessive forms of DEB exist and to some extent paradigms for genotype-phenotype correlation have emerged. Dominant forms of DEB usually result from heterozygous missense GS mutations exerting a dominant negative effect, while recessive forms of DEB result from homozygous or compound heterozygous nonsense, frameshift or splice site mutations. While missense GS mutations are typically associated with dominant forms of DEB, non-GS mutations are also associated with DDEB, as well as milder forms of RDEB (Dang and Murrell, 2008; Almaani *et al.*, 2011; Hovnanian *et al.*, 1997; Varki *et al.*, 2007; Whittock *et al.*, 1999).

In this Chapter, 16 novel non-GS mutations are identified, expanding on the current *COL7A1* non-GS mutations database. The significance of these missense mutations on DEB phenotype was studied in the aim of refining genotype-phenotype correlation, which will have important implications for diagnosis and genetic counselling.

5.2 Patients and Methods

For this study I collected and evaluated DNA mutation analysis data retrospectively, via the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory database in London. Blood samples from patients with clinically suspected or histologically proven DEB (and their parents if available), were provided historically by local, national and international referrers. Patient

ethnicities included white Caucasian, Middle-Eastern, South American, South-East Asian and Asian. Prior to my work on this thesis, the DNA extraction and *COL7A1* mutation analysis had been performed by Dr. Lu Liu at the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory, as part of the patients' routine clinical care in most cases. However, for individuals linked to separate research projects, DNA screening was undertaken with Ethics' Committee approval (St Thomas' Hospital Ethics' committee; 07/H0802/104) with informed consent and carried out in accordance with the Declaration of Helsinki principles. DNA extraction and sequencing was performed as discussed in Chapter 2. Polymorphisms were ruled out by excluding mutations in 100 controls and performing *in silico* analysis where necessary.

5.3 Results and Discussion

This study revealed that non-GS mutations represent around 30% of the total *COL7A1* missense substitution mutations. However, only 7% occur in dominant DEB compared to 40% of recessive DEB cases (Table. 5.1 and Figure 5.1) (Almaani *et al.*, 2011). This is a reflection of the uniquely high glycine content of *COL7A1*, which is arranged, in Gly-*X*-*Y* repeats along the triple helical domain (Whittock *et al.*, 1999; Hovnanian *et al.*, 1997; Ryoo *et al.*, 2001; Dang and Murrell, 2008). The glycine residues are centred within the *COL7A1* triple helix while the *X*-*Y* residues are positioned exteriorly (Persikov *et al.*, 2004; Fritsch *et al.*, 2009). As a consequence, glycine amino acids are integral to the triple helical stability and suprastructural conformation while alterations in non-glycine amino acids are likely to have a less deleterious effect (Fritsch *et al.*, 2009; Persikov *et al.*, 2004). Therefore, the higher

association of non-GS mutations with RDEB compared to DDEB is related to the significantly higher occurrence of GS mutations in cases of DDEB rather than a more detrimental effect of non-GS mutations. Also, the association of non-GS mutations with a splice site or loss of function mutation on the other allele will account for the more severe outcome in cases of RDEB. However, non-GS mutations, particularly in highly conserved or critical regions, have also been found to affect helix formation, protein folding, resistance to protease degradation, intracellular transport and secretion, fibroblast adhesion, keratinocyte migration as well as assembly into anti-parallel dimers and anchoring fibrils (van den Akker *et al.*, 2011; Chen *et al.*, 2002a; Brittingham *et al.*, 2005; Woodley *et al.*, 2008; Kern *et al.*, 2006; Hovnanian *et al.*, 1997; Chen *et al.*, 2001).

The majority of pathogenic non-GS mutations result from the substitution of arginine, a large, hydrophilic amino acid, leading to loss of an ionic charge or the introduction of a bulky amino acid at external helical surface (Hovnanian *et al.*, 1997). Through alterations in the size and charge of amino acids, non-GS mutations result in conformational changes in collagen VII leading to deleterious functional effects as outlined above. The substitutions which mainly involve arginine residues (~35% of cases) and proline to a lesser extent (~20% of cases) (Table. 5.2) lead to mild forms of RDEB when inherited on both alleles, however, when inherited *in trans* with non-sense or frameshift mutations, some non-GS mutations lead to a more severe phenotype (RDEB-sev gen). Interestingly, two homozygous non-GS mutations result in RDEB-sev gen; p.Arg2008Gly and p.Arg2063Trp (Hovnanian *et al.*, 1997). This could be explained by the nature of these

mutations, particularly the substitution of the positively charged hydrophilic arginine residue by a larger hydrophobic tryptophan residue in the p.Arg2063Trp mutation with more deleterious effects on *COL7A1* ultrastructural conformation (Woodley *et al.*, 2008; Wessagowit *et al.*, 2005). The position of these non-GS mutations in relation to critical or highly conserved areas might also play a role in phenotype expression. Both p.Arg2008Gly and p.Arg2063Trp mutations in exons 73 and 74, respectively, lie in close proximity to the 'hinge region' with potential conformational changes and increased sensitivity to protease degradation (Woodley *et al.*, 2008; Wessagowit *et al.*, 2005). However, the homozygous mutation p.Arg2008Gly was also found to result in RDEB-i suggesting that other factors might also influence phenotype expression (van den Akker *et al.*, 2011).

Table 5.1 Reported and novel non-glycine substitution mutations in the collagen VII gene.

	Allele 1				Allele 2				
Diagnosis	**Exon**	**cDNA**	**Type**	**Protein**	**Exon/ Intron**	**cDNA**	**Type**	**Protein**	**Reference**
RDEB-sev gen	1	c.3G>T or G>A	MS	p.Met1Ile	54	c.4997dupG	PTC	p.Pro1668AlafsX4	(van den Akker *et al.*, 2009)
RDEB-pr RDEB-na	1	c.82A>G	MS	p.Arg28Gly	92	c.7097G>C	MS	p.Gly2366Arg	(Pruneddu *et al.*, 2011)
RDEB-O	2	c.112G>T	MS	p.Asp38Tyr	16	c.2157G>A	PTC	p.Trp719Stop	www.col7a1.org
RDEB-pr	2	c.130G>A	MS	p.Asp44Asn	16	c.8569G>T	PTC	p.Glu2857Stop	(Makino *et al.*, 2012)
RDEB-sev gen	2	c.143C>T	MS	p.Ser48Pro[a]	27	c.3625del11	PTC	p.Ser1209LeufsX6	(Jiang *et al.*, 2005)
RDEB-pr	2	c.151C>G	MS	p.Arg51Gly	98	c.7474C>T	PTC	p.Arg2492Stop	(Drera *et al.*, 2006)
RDEB-sev gen	2	c.238G>C	MS	p.Ala80Pro	27	c.3631C > T	PTC	p.Gln1211Stop	(Varki *et al.*, 2007)
RDEB-O	2	c.245T>G	SS	p.Val82Gly	ND	ND	ND	ND	(Wessagowit *et al.*, 2005)
RDEB-O	3	c.410A>G	MS	**p.Lys137Arg**	72	c.5869C>T	MS	p.Arg1957Trp	**This study**
RDEB-O	3	c.425A>G	SS	p.Lys142Arg	95	c.7344G>A	SS	p.Val2448Val	(Gardella *et al.*, 1996)
DDEB-gen	7	c.884G>A	MS	**p.Arg295Gln**	108	c.8045 A>G	SS	predicted skipping of exon 108 (in-frame; 63aa)	**This study**
RDEB-O	17	c.2126T>C	MS	**p.Val709Ala**	13	c.1732C>T	PTC	p.Arg578Stop	**This study**

DDEB	17	c.2278G>A	MS	p.Val760Met	/	/	/	/	(Varki *et al.*, 2007)
RDEB-O	20	c.2657G>C	MS	p.Arg886Pro	4	c.497insA	PTC	p.Val168GlyfsX12	(Posteraro *et al.*, 2005)
RDEB-O	21	c.2729G>C	MS	p.Arg910Pro	3	c.425A>G	SS	p.Lys142Arg	(van den Akker *et al.*, 2009)
RDEB-O	22	c.2936T>G	MS	**p.Val979Gly**	IVS52	4935+2T>G	ND	ND	**This study**
RDEB	22	c.2969G>A	MS	p.Arg990Gln	73	c.6023G>A	MS	p.Arg2008His	(Nakano *et al.*, 2007)
RDEB-O	25	c.3301C>T[b]	MS	p.Arg1101Trp	34	c.4027C>T	PTC	p.Arg1343Stop	**This study**
RDEB-O	25	c.3359G>A	SS	p.Arg1120Lys	ND	ND	ND	ND	(Wessagowit *et al.*, 2005)
RDEB-O	28	c.3749A>C	MS	p.Tyr1250Ser	73	c.6084C>T	MS	p.Pro2029Ser	(Ryoo *et al.*, 2001)
RDEB-sev gen	30	c.3809C>T	MS	p.Pro1270Leu	IVS5	c.682+1G>A	SS	predicted retention of intron 5 + p.Pro228fsX32	www.col7a1.org
RDEB-pr	31 31	c.3847C>T c.3889G>A	MS MS	**p.Pro1283Ser** **p.Glu1297Lys**[c]	3	c.425insA	PTC	p.Lys142fsX42	**This study**
RDEB-O	33	c.4011G>A	SS	p.Pro1337Pro	104	c.7769G>A	MS	p.Gly2590Asp	(van den Akker *et al.*, 2009)
RDEB-O	35	c.4118C>T	SS	p.Ser1373Leu	116	c.8569G>T	PTC	p.Glu2857Stop	(Mayama *et al.*, 1999)
RDEB-sev gen	40	c.4373C>T	MS	p.Pro1458Leu[d]	103	c.7723G>A	MS	p.Gly2575Arg	(Varki *et al.*, 2007)

RDEB-sev gen	43	c.4489C>T	MS	**p.Arg1497Cys**	80	c.6527insC	PTC	p.Gly2177TrpfsX11 3	**This study**
DEB	46	c.4613G>A	MS	p.Arg1538His	ND	ND	ND	ND	(Makino *et al.*, 2012)
RDEB	47	c.4666A>G	MS	**p.Lys1556Glu**	IVS22	2858-7del82	PTC	ND	**This study**
RDEB-sev gen	51	c.4879G>A	MS	p.Val1627Ile	116	c.8569G>T	PTC	p.Glu2857Stop	(Cho *et al.*, 2009)
RDEB-O	54	c.5033C>T	SS	p.Pro1678Leu	3	c.425 A>G	SS	p.Lys142Arg	(Wertheim-Tysarowska *et al.*, 2012)
RDEB-pt	55	c.5096C>T	MS	p.Pro1699Leu	IVS2	c.267-1G>C	SS	predicted skipping of exon 3 (out of frame; 160aa)	(Gardella *et al.*, 2002)
RDEB-O	61	c.5314C>T	MS	p.Arg1772Trp	104	c.7786delG	PTC	p.Gly2596ValfsX49	(Whittock *et al.*, 1999)
RDEB-O	63	c.5440C>T	MS	p.Arg1814Cys	113	c.8386C>T	PTC	p.Gln2796Stop	(Dang *et al.*, 2007)
DEB	69	c.5771A>C	MS	p.Gln1924Pro	ND	ND	ND	ND	(Pulkkinen and Uitto, 1999)
RDEB-O	70	c.5819C>T	MS	p.Pro1940Leu[e]	IVS42	c.4482+1G>A	SS	predicted skipping of exon 42 (in frame; 45aa)	(Varki *et al.*, 2007)
RDEB-O	70	c.5820G>A	SS	p.Pro1940Pro	34	c.4039G>C	MS	p.Gly1347Arg	(Terracina *et al.*, 1998)
RDEB-O	72	c.5869C>T	MS	**p.Arg1957Trp**	3 54	c.410 A>G or c.5047 C>T	MS PTC	p.Lys137Arg or p.Arg1683Stop	**This study**

RDEB-O	72	c.5870G>A	MS	p.Arg1957Gln	IVS81	c.6573+1G>C	PTC	p.Gly2192fsX106	(Sawamura *et al.*, 2005)
RDEB-acral	72	c.5942A>G	MS	p.Lys1981Arg	4	c.497dupA	PTC	p.Val168GlyfsX12	(Jerabkova *et al.*, 2010)
DEB	73	c.6004C>T	MS	p.Arg2002Cys	ND	ND	ND	ND	(Pulkkinen and Uitto, 1999)
RDEB-sevgen	73	c.6022C>G	MS	p.Arg2008Gly	73	c.6072C>G	MS	p.Arg2008Gly	(Hovnanian *et al.*, 1997)
RDEB-O	73	c.6022C>T	MS	p.Arg2008Cys	70	c.5818delC	PTC	p.Pro1940ArgfsX65	(Kon *et al.*, 1998)
RDEB-O	73	c.6080C>T	MS	p.Pro2027Leu	13	c.1732C>T	PTC	p.Arg578Stop	(Kern *et al.*, 2009a)
RDEB-O	73	c.6084C>T	MS	p.Pro2029Ser	28	c.3749A>C	MS	p.Tyr1250Ser	(Ryoo *et al.*, 2001)
RDEB	73	c.6134C>T	MS	p.Pro2045Leu	3	c.425 A>G	SS	p.Lys142Arg	(Wertheim-Tysarowska *et al.*, 2012)
RDEB	73	c.6023G>A	MS	p.Arg2008His	22	c.2969G>A	MS	p.Arg990Gln	(Nakano *et al.*, 2007)
RDEB-O	73	c.6176A>G	MS	p.Glu2059Gly	73	c.6176A>G	MS	p.Glu2059Gly	(Kern *et al.*, 2006)
RDEB	73	c.6180G>C	SS	p.Arg2060Ser	5	c.553C>T	PTC	p.Arg185Stop	(van den Akker *et al.*, 2009)
RDEB-i	74	c.6187C>G	MS	p.Arg2063Gly	6	c.706C>T	PTC	p.Arg236Stop	(Hovnanian *et al.*, 1997)
RDEB-sevgen	74	c.6187C>T	MS	p.Arg2063Trp	74	c.6187C>T	MS	p.Arg2063Trp	(Hovnanian *et al.*, 1997)

RDEB-i	74	c.6205C>T	MS	p.Arg2069Cys	3	c.425 A>G	SS	p.Lys142Arg	(Kahofer *et al.*, 2003)
DDEB-O DDEB-BDN	74	c.6216A>G	SS	**p.Gln2072Arg**	/	/	/	/	**This study**
RDEB-O[f]	80	c.6510G>T	MS	p.Gln2170Tyr	80	c.6510G>T	MS	p.Gln2170Tyr	(van den Akker *et al.*, 2009)
RDEB-O	84	c.6677T>A	MS	p.Val2226Glu	84	c.6685C>T	MS	p.Pro2229Ser	(Ryoo *et al.*, 2001)
RDEB-O	84	c.6685C>T	MS	p.Pro2229Ser	84	c.6677T>A	MS	p.Val2226Glu	(Ryoo *et al.*, 2001)
RDEB-O	84	c.6695C>G	MS	p.Pro2232Arg	84	c.6697G>C	MS	p.Gly2233Cys	(Ryoo *et al.*, 2001)
RDEB-O	86	c.6767C>T	MS	**p.Pro2256Leu**	86	c.6767 C>T	MS	p.Pro2256Leu	**This study**
DDEB-pr	87	c.6846G>C	SS	p.Leu2282Leu	/	/	/	/	(Covaciu *et al.*, 2011)
DDEB-gen	87	c.6899 A>G	SS	p.Gln2300Arg	/	/	/	/	(Kern *et al.*, 2006)
RDEB-O	88	c.6927G>C	MS	p.Lys2309Asn	111	c.8253-54dupAG	PTC	p.Val2752GlufsX34	(Kern *et al.*, 2009a)
RDEB-O	94	c.7245G>A	SS	p.Met2415Ile[g]	large genomic deletion which removes the entire *COL7A1* gene				(Titeux *et al.*, 2006)
RDEB-sev gen	95	c.7268A>G	MS	p.Glu2423Gly	80	c.6528insC	PTC	p.Gly2177TrpfsX113	(Varki *et al.*, 2007)
RDEB	95	c.7313C>G	MS	p.Pro2438Arg[h]	ND	ND	ND	ND	(Rodriguez *et al.*, 2012)

RDEB-sev gen	95	c.7313C>T	MS	p.Pro2438Leu[i]	73	c.6082G>A	MS	p.Gly2028Arg	(Varki *et al.*, 2007)
RDEB-O	95	c.7344G>A	SS	p.Val2448Val	3	c.425A>G	SS	p.Lys142Arg	(Gardella *et al.*, 1996)
RDEB-pt	103	c.7738C>T	MS	p.Arg2580Cys	IVS90	c.7023+1G>A	SS	ND	www.col7a1.org
DDEB-gen	105	c.7846G>A	MS	**p.Val2616Ile**	/	/	/	/	**This study**
RDEB-i	105	c.7864C>T	MS	p.Arg2622Trp	19	c.2482delCT	PTC	p.Ser828CysfsX37	(Gardella *et al.*, 2002)
RDEB-sev gen	105	c.7865G>A	MS	p.Arg2622Gln	ND	ND	ND	ND	(Varki *et al.*, 2007)
RDEB-i	106	c.7882C>T	MS	p.Arg2628Trp	10	c.1332C>A	PTC	p.Tyr444Stop	(Chiaverini *et al.*, 2010)
DDEB-O	108	c.8045A>G	SS	**p.Lys2682Arg**	/	/	/	/	**This study**
RDEB-pr	110	c.8206G>A	MS	p.Glu2736Lys	3	c.425A>G	SS	p.Lys142Arg	(Schumann *et al.*, 2008)
DDEB	113	c.8371C>T	MS	p.Arg2791Trp	/	/	/	/	(Whittock *et al.*, 1999)
RDEB	113	c.8393T>G	MS	p.Met2798Arg	ND	ND	ND	ND	(Rodriguez *et al.*, 2012)
RDEB-O	113	c.8393T>A	MS	p.Met2798Lys	113	c.8393T>A	MS	p.Met2798Lys	(Christiano *et al.*, 1993)
RDEB	115	c.8486A>G	MS	**p.His2829Arg**	ND	ND	ND	ND	**This study**
RDEB-O	117	c.8627G>T	MS	p.Cys2875Phe[j]	33	c.4018C>T	PTC	p.Arg1340Stop	(Sawamura *et al.*, 2005)

RDEB-O	117	c.8626T>C	MS	**p.Cys2876Arg**	12	c.1564 C>T	PTC	p.Gln522Stop	**This study**
RDEB-O	117	c.8644G>A	MS	p.Glu2882Lys	116	c.8569G>T	PTC	p.Glu2857Stop	(Hanafusa *et al.*, 2012)
RDEB-O	117	c.8764C>T	MS	p.Arg2922Cys	54	c.5047C>T	PTC	p.Arg1683Stop	(Kern *et al.*, 2009a)
RDEB-sev gen	117	c.8780G>A	MS	p.Arg2927His	52	c.4919delG	PTC	p.Gly1640ValfsX70	(Varki *et al.*, 2007)
RDEB-O	117	c.8780G>C	MS	**p.Arg2927Pro**	31	c.3840delC	PTC	p.Gly1281ValfsX44	**This study**

DDEB: dominant dystrophic epidermolysis bullosa; DDEB-BDN: bullous dermolysis of the newborn; DDEB-gen: generalized; DDEB-pr: pruriginosa; RDEB: recessive dystrophic epidermolysis bullosa; RDEB-sev gen: severe generalized; RDEB-O: other; RDEB-BDN: bullous dermolysis of the newborn; RDEB-na: nail dystrophy; RDEB-pr: pruriginosa; RDEB-i: inversa, IVS, intervening sequence (intron); MS: missense; PTC: premature termination codon; ND: not determined; SS: splice site, bold: new mutations. [a]p.Ser48Pro but corrected nomenclature should be: p.Ser48Phe, [b]inherited with the c.5504delA on the same allele, [c]inherited on the same allele, [d]inherited with the p.Gly2251Ala mutation on the same allele, [e]inherited with the c.5015delA mutation on the same allele, [f]homozygous carrier of p.Gln2170Stop demonstrating revertant mosaicism in an unaffected patch of skin carrying a homozygous p.Gln2170Tyr mutation, [g]can lead to a delayed PTC p.Gln2417SerfsX57, [h]possible polymorphism, [i]inherited with the p.Gly1580Asp mutation on the same allele, [j]p.Cys2875Phe but corrected nomenclature should be: p.Cys2876Phe.

Figure 5.1 A schematic outlining the position of non-GS mutations within *COL7A1*. Novel dominant and recessive non-GS mutations identified in this study are highlighted in red and blue respectively.

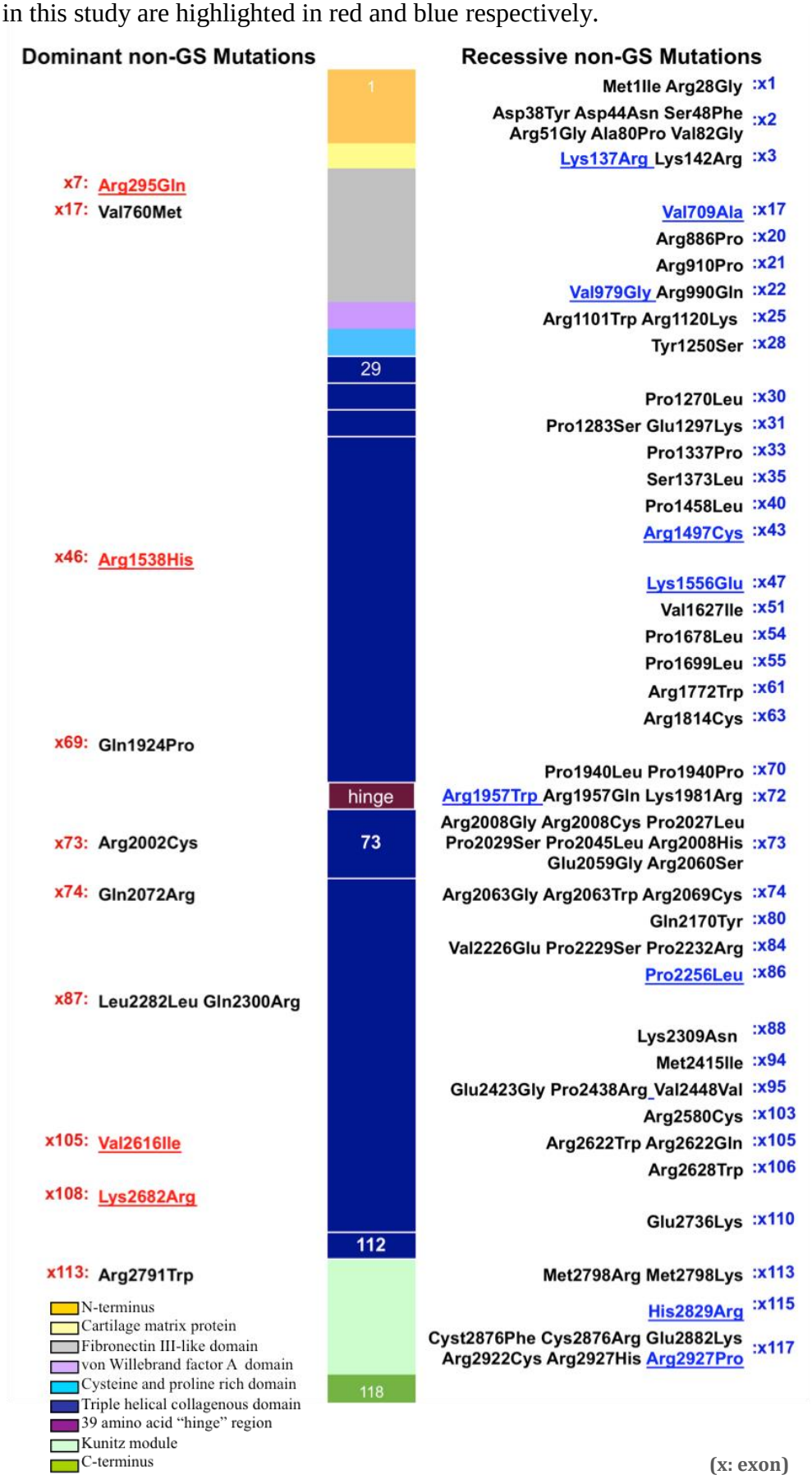


Table 5.2 The type and frequency of *COL7A1* non-glycine substitution mutations identified in this study. Arginine was the most frequently substituted amino acid. It was also the most likely to replace other amino acids along with leucine.

Amino Acid	Symbol	As the original amino acid	As a substitution
Arginine	Arg	30	12
Cysteine	Cys	2	7
Proline	Pro	16	7
Valine	Val	8	1
Tryptophan	Trp	0	7
Glutamine	Gln	4	4
Aspartic Acid	Asp	2	0
Glutamic acid	Glu	5	2
Histidine	His	1	3
Methionine	Met	4	1
Lysine	Lys	6	4
Serine	Ser	2	5
Tyrosine	Tyr	1	2
Phenylalanine	phe	0	2
Isoleucine	Ile	0	4
Glycine	Gly	not included	8
Alanine	Ala	1	1
Leucine	Leu	1	11
Asparagine	Asn	0	2
Total		84	84

Furthermore, although the majority of non-glycine mutations are missense mutations some alter splicing. However, none of these mutations resulted in a more severe phenotype (Wessagowit *et al.*, 2005). In addition 2 novel non-GS splice site mutations were identified in this study that resulted in DDEB; p.Gln2072Arg and p.Lys2682Arg. The mutation p.Gln2072Arg is predicted to result in skipping of exon 74 leading to mild DDEB (DDEB-O). The proband's mother is also heterozygous for this mutation which resulted in transient blistering for 2 years after birth and mild nail dystrophy. The dominantly inherited mutation p.Lys2682Arg in exon 108 also resulted in DDEB-O. This mutation disrupts the donor splice site, resulting in a leaky site as predicted by in *silico* analysis. Another previously reported splice site mutation, p.Gln2300Arg, which is predicted to result in skipping of exon 87, was also found in this study cohort. Interestingly, this mutation, which was present in 3 generations, was phenotypically heterogeneous. The grandmother had dominant EB-pr and the granddaughter demonstrated skin fragility at birth, while the daughter was asymptomatic. However, as the splicing anomaly is predicted to result in a leaky site, clinical heterogeneity is not unexpected.

In addition to the type of mutation, the location of the non-GS mutations could play a role in determining phenotype expression. Mutations in the non-collagenous domains, NC1 and NC2, could affect collagen VII attachment to the basement membrane zone and triple helical formation and assembly respectively (Chen *et al.*, 2001; Woodley *et al.*, 2008). However, non-GS mutations in these two domains are quite variable resulting in DDEB, RDEB-O, as well as RDEB-sev gen. Moreover, mutations in close proximity to the highly conserved cysteine residues can potentially interfere with disulphide

bonding necessary for anti-parallel dimer formation (Dang and Murrell, 2008; van den Akker *et al.*, 2011; Brittingham *et al.*, 2005; Chen *et al.*, 2001). The mutations p.Arg2622Trp, p.Arg2622Gln and p.Arg2628Trp are in close proximity to the only cysteine residue in the triple helix collagenous domain (THC) at position 2634 which can lead to unstable antiparallel dimers or alteration in the *COL7A1* suprastructural conformation (van den Akker *et al.*, 2011; Christiano *et al.*, 1994d). On the other hand the p.Met2798Lys, lies in close proximity to the cysteine residues in the NC2 domain necessary for triple helical and antiparrallel dimer formation (Chen *et al.*, 2001). However, the phenotype for mutations near cysteine residues is also quite variable involving RDEB-O, RDEB-pr and RDEB-I. Furthermore, only one cysteine substitution mutation has been reported in *COL7A1*; p.Cys2875Phe, which is inherited *in trans* with a loss of function mutation; p.Arg1340Stop, resulting in mild RDEB (Sawamura *et al.*, 2005). This study adds a second *COL7A1* cysteine substitution mutation, p.Cys2876Arg that is also inherited *in trans* with the nonsense mutation p.Gln522Stop and results in RDEB-O.

Non-GS mutations within the triple helix collagenous domain, particularly in relation to the hinge region, can affect the stability of the triple helix, resistance to protease degradation as well as its suprastructural conformation and flexibility. There are also effects on collagen VII secretion with subsequent intracellular retention of collagen VII (Chen *et al.*, 2002a; van den Akker *et al.*, 2011; Woodley *et al.*, 2008). However, phenotype expression of non-GS mutations could not be explained solely by location as different amino acid substitutions in the same position lead to varying phenotypes (p.Arg2008Gly; RDEB-sev gen, p.Arg2008Cys; RDEB-O, p.Arg2063Gly;

RDEB-I, p.Arg2063Trp; p.Arg2622Trp; RDEB-I, p.Arg2622Gln; RDEB-sev gen, RDEB-sev gen, p.Arg2927His; RDEB-sev gen, p.Arg2927Pro; RDEB-O). Furthermore specific DEB subtypes are not related to a particular amino acid substitution as one study revealed that the size, water avidity and polarity of the amino acids substituting arginine in RDEB-I, had no bearing on the clinical phenotype (van den Akker *et al.*, 2011). It is likely however, that phenotypic heterogeneity could, in part be explained by genetic and epigenetic modifiers including variable splicing and degradation, large genomic deletions and silent mutations as well as effects of endoplasmic reticulum stress and mutant proteins on apoptosis and gene expression (Covaciu *et al.*, 2011; Nissim-Rafinia and Kerem, 2005; Bateman *et al.*, 2009; Knaup *et al.*, 2012; Kern *et al.*, 2009a; Titeux *et al.*, 2006).

In conclusion the heterogeneity of non-GS mutations is reflected by a variable phenotypic constellation. The type or location of the non-GS mutations might predict the functional consequences and resultant phenotypic severity; however, it is more likely that a combination of factors including potential genetic and epigenetic modifiers modulate collagen VII expression and the resultant phenotype. Advances in functional genomics that explore the dynamic interplay between genomics, proteomics and subsequent clinical expression is likely to yield a more comprehensive understanding of genotype-phenotype correlation in EB. Coupled to bioinformatic advances, this will help to provide biologically meaningful patterns of disease expression useful for translational medicine.

Chapter 6

Phenotype modulation by genetic modifiers: The role of matrix metalloproteinase 1 (MMP-1) on the epidermolysis bullosa pruriginosa phenotype

Abstract

Epidermolysis bullosa pruriginosa (EB-pr is an unusual subtype of DEB characterised by severe pruritus that leads to striking skin changes resembling nodular prurigo or hypertrophic lichen planus. The *COL7A1* molecular pathology underlying EB-pr is similar to that seen in other non-pruritic (non EB-pr) variants of DEB but the reason for the striking phenotypic heterogeneity is still not clear. In this study, the incidence of a common functional polymorphism in the matrix metalloproteinase 1 gene promoter (1G or 2G at nucleotide -1607) is assessed in patients with EB-pr (n=27) compared to non-itchy dominant DEB (n=23), recessive DEB (n=25) and normal controls (n=50). The 2G allele was previously found to result in increased matrix metalloproteinase 1 activity, which leads to increased degradation of collagen VII and a more severe RDEB phenotype. The aim of the study was to assess if the 2G allele could explain the phenotypic heterogeneity encountered in other forms of DEB particularly EB-pr It might be that the MMP-1 induced increase in collagen VII degradation, triggers an inflammatory response that leads to the pruritus characteristic of EB-pr. The frequency of the 2G allele in EB-pr patients was 46.3%, greater than in the control group (42.0%) but less than in non-itchy dominant DEB (52.1%) or recessive DEB (62.0%). However, none of these differences were statistically significant apart from recessive DEB compared to controls (p=0.02). In conclusion, this study expands on the current database of *COL7A1* mutations leading to EB-pr and reveals that a common functional polymorphism in the MMP-1 gene promoter does not contribute to the unique pruritic EB-pr variant, the pathophysiology of which remains uncertain.

6.1 Introduction

As discussed in previous Chapters there is often overlap between the various sub-categories of DEB particularly in the dominantly inherited group where inter- and intra-familial phenotypic heterogeneity is frequently observed (Ee *et al.*, 2007; Mellerio *et al.*, 1999; Nakamura *et al.*, 2004; Murata *et al.*, 2000; Bodemer *et al.*, 2003). Although the type, combination and location of mutations within the *COL7A1* gene could provide insight into the clinical outcome, phenotypic variability suggests that genetic, epigenetic or environmental modifiers might play a role in this heterogeneity.

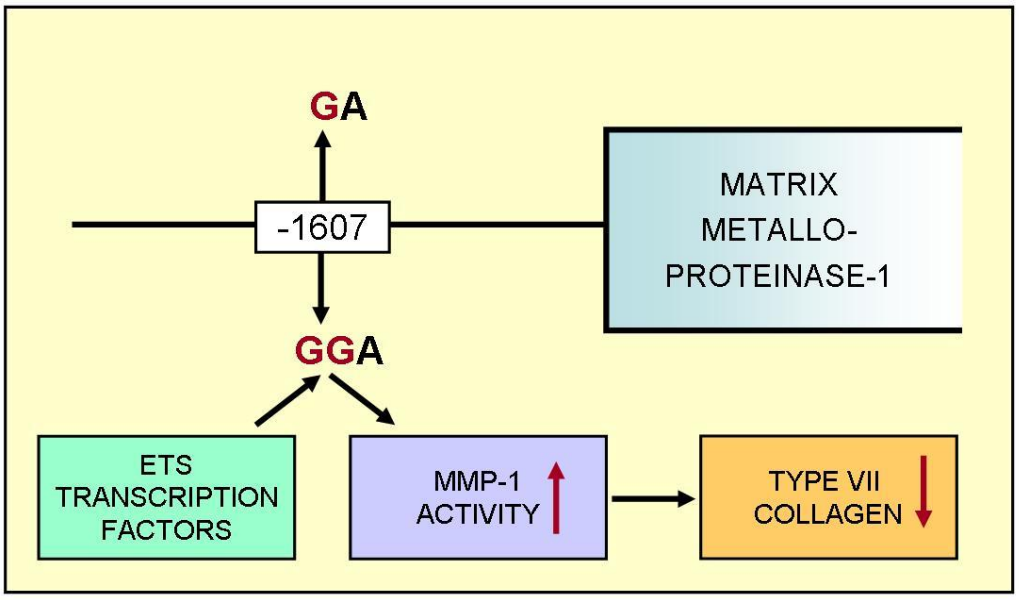
One particular subtype of DEB, EB-pr, has been difficult to categorise. This unique form of DEB has clinical features overlapping with pretibial DEB and is associated with intense pruritus (McGrath *et al.*, 1994). The resultant clinical features are striking and resemble lichen simplex chronicus, nodular prurigo, immunobullous diseases, hypertrophic lichen planus or even dermatitis artefacta (McGrath *et al.*, 1994; Lee *et al.*, 1997; Mellerio *et al.*, 1999). EB-pr is usually inherited as an autosomal dominant disease but may be recessive in some cases (McGrath *et al.*, 1994; Mellerio *et al.*, 1999; Drera *et al.*, 2006; Schumann *et al.*, 2008). However, the nature of the underlying *COL7A1* mutations is not different from other forms of DEB (Mellerio *et al.*, 1999; Lee *et al.*, 1997; Drera *et al.*, 2006; Dang *et al.*, 2007; Ee *et al.*, 2007; Jiang *et al.*, 2002; Murata *et al.*, 2000; Nakamura *et al.*, 2004; Whittock *et al.*, 1999) with even some identical mutations resulting in variable forms of EB including EB-pr (Mellerio *et al.*, 1999; Lee *et al.*, 1997; Drera *et al.*, 2006; Dang *et al.*, 2007; Ee *et al.*, 2007; Jiang *et al.*, 2002; Murata *et al.*, 2000; Nakamura *et al.*, 2004; Whittock *et al.*, 1999).

Studies exploring the influence of potential modifiers on the pruritic EB-pr phenotype including increased IgE levels; atopy; renal, hepatic and thyroid dysfunction; iron deficiency, interleukin-31 and filaggrin mutations have so far failed to establish a definite link (Mellerio *et al.*, 1999; Drera *et al.*, 2006; Schumann *et al.*, 2008; Ee *et al.*, 2007; Lapinski *et al.*, 1998; Nagy *et al.*, 2010; Ren *et al.*, 2008). Therefore any contributing metabolic, genetic, epigenetic, or environmental factors are yet to be determined.

However, a recent study by Titeux *et al.* has demonstrated a significant increase in the frequency of a GG single nucleotide polymorphism (SNP) in the promoter area of the zinc metalloproteinase MMP-1 gene in patients with the most severe type of RDEB; RDEB-sev gen (Titeux *et al.*, 2008). The polymorphism, which is found at position -1607 within the *MMP-1* promoter area occurred as either 1G or 2G, both of which have been shown to occur with similar frequency in control populations (Ye *et al.*, 2001; Zinzindohoue *et al.*, 2005). However, the 2G variant at this site was shown to create an ETS (E26 transformation specific) transcription factor binding site, 5'-GGA-3', resulting in a 2-fold transcriptional up regulation of *MMP-1* (Rutter *et al.*, 1998) (Figure 6.1). The latter is a collagenase secreted by basal keratinocytes and dermal fibroblasts and is of particular interest as collagen VII acts as one of its substrates (Seltzer *et al.*, 1989). This is in keeping with the finding that although the synthesis of collagen VII was found to be normal by Titeux *et al.*, there was a reduction at the protein level due to increased MMP-1 induced collagen VII degradation. No significant difference in collagen VII expression was found between the 2G/1G and 2G/2G SNP variants (Titeux *et al.*, 2008). Other studies also observed increased levels of MMP-2, 3 and 9 and a reduced

level of tissue inhibitors of metalloproteinases (TIMPs) in the more severely affected RDEB cases (Bodemer *et al.*, 2003).

Figure 6.1 The 1G/2G polymorphism in the MMP- 1 gene promoter is located at nucleotide -1607 from the *MMP1* transcription site. The 2G polymorphism creates an ETS (E26 transformation specific) transcription factor binding site which increases the transcriptional activity of *MMP1*. This leads to an increased degradation of collagen VII.



In this study I sought to determine if the phenotypic heterogeneity between classical DDEB and EB-pr could be explained by the *MMP-1* promoter polymorphism. The variability in disease expression could in part be explained by the MMP-1 induced increase in collagen VII degradation that triggers secondary inflammatory changes and cytokine production leading to the pruritus characteristic of EB-pr (Mellerio *et al.*, 1999; Drera *et al.*, 2006).

6.2 Patient selection and methods

DNA samples from individuals with EB-pr (n=27), non EB-pr dominant DEB (n=23), recessive DEB (n=25), as well as unaffected, unrelated and ethnically matched control subjects (n=50) were analysed in this study. The controls were not age- or sex- matched. Patient ethnicities included white Caucasian, Middle-Eastern, South American, South-East Asian and Asian.

I sequenced the *MMP1* promoter region corresponding to the predicted 1G/2G polymorphism in all DEB and control samples using the following primers: forward primer 5'-gtggaagcttacacctataatcccaacactc-3' (-4008 bp to -3988 bp; GenBank No NM002421) and reverse primer 5'-ctgcctggtaccctattgcgatagcaccatggc-3' (-511 bp to -543 bp) using the Sanger sequencing technique and with PCR amplification conditions as described previously (Mellerio *et al.*, 1998). I compared the frequency of the two alleles between the different groups using Fisher's exact test.

The blood samples for DNA mutation analysis were provided historically by local, national and international referrers to the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory in London. Prior to my work on this thesis, the DNA extraction and *COL7A1* mutation analysis had

been performed by Dr. Lu Liu at the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory, as part of the patients' routine clinical care in most cases. However, for individuals linked to separate research projects, DNA screening was undertaken with Ethics' Committee approval (St Thomas' Hospital Ethics' committee; 07/H0802/104) with informed consent and carried out in accordance with the Declaration of Helsinki principles. Genomic DNA extraction and *COL7A1* mutation screening was performed as described in Chapter 2.

6.3 Results

In the cohort studied, all 27 EB-pr subjects (19 families) included had dominant DEB resulting from heterozygous *COL7A1* glycine substitution mutations (6 novel), while the 23 non EB-pr dominant cases (20 families) were caused by 13 heterozygous glycine substitution mutations (six novel) and a new splice site mutation; IVS37+1G>T (Figure 6.2). There was no significant difference in the type or location of the *COL7A1* GS mutations between the dominant EB-pr and non EB-pr groups. In addition, phenotypic heterogeneity was observed, as evidenced by the variable disease expression of the heterozygous GS mutation p.Gly2043Arg observed in this study. Out of 12 patients with this mutation, 4 patients had EB-pr while 8 had non EB-pr dominant DEB (Table 6.1).

On a molecular level, the incidence of the MMP1 polymorphism in the various clinical subgroups was analysed. The major allele in the control group was 1G (58.0%) while the minor allele was 2G (42.0%). In the EB-pr group,

the frequency of the 1G allele was 53.7% and of 2G was 46.3%, whereas in the non EB-pr dominant DEB subjects the frequency of 1G and 2G alleles was 47.8% and 52.1% respectively. 2G was the major allele in the recessive DEB group (62.0%) (Table 6.2). There was no significant difference in allelic frequency between EB-pr and controls (p=0.61), EB-pr and non EB-pr dominant DEB (p=0.56), or non EB-pr dominant DEB and controls (p=0.25). However, in keeping with published data the allelic frequency was significantly different between the recessive DEB group and controls (p=0.02): where the frequency of the 2G allele was found to be higher in the RDEB group.

Figure 6.2 *COL7A1* mutation analysis in individuals with dominant DEB in this study. Previously unreported mutations are highlighted in red boxes.

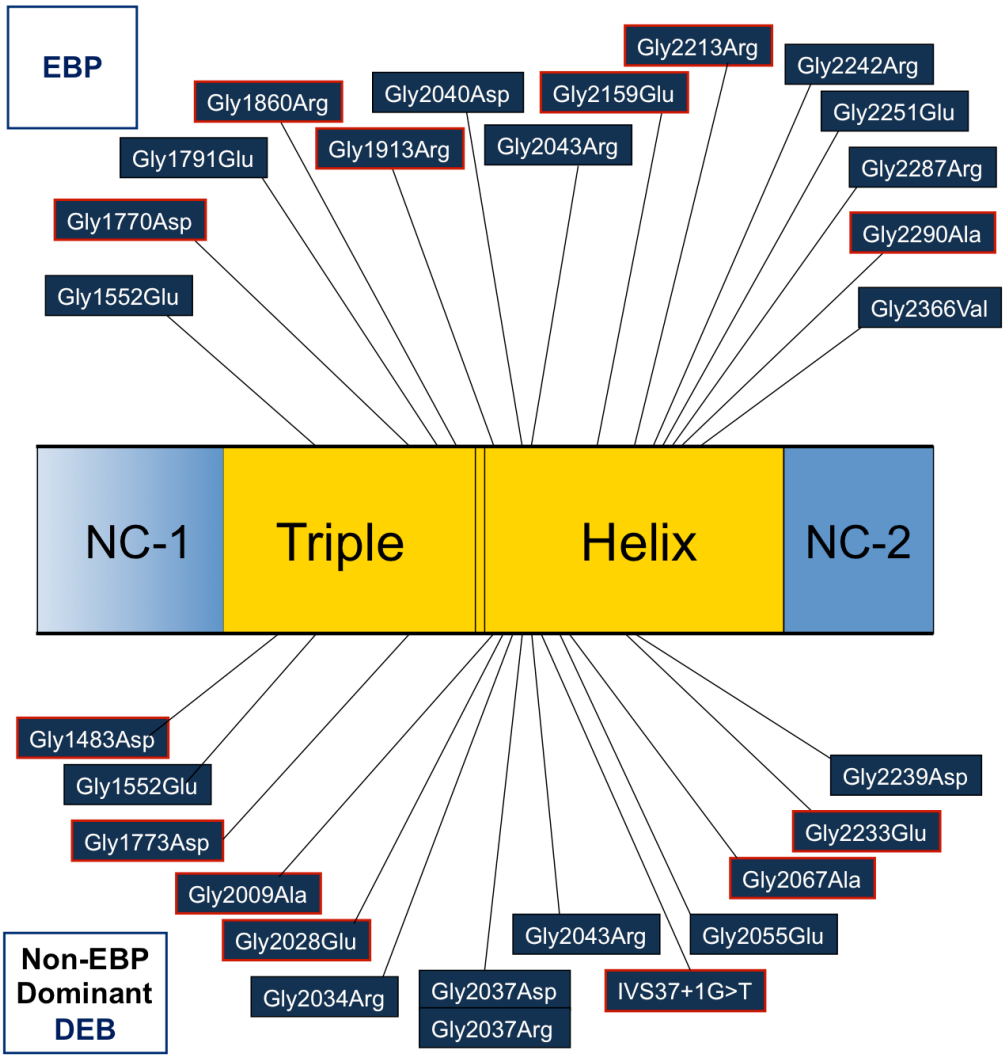


Table 6.1 *COL7A1* mutations and *MMP1* promoter polymorphisms associated with the various DEB subgroups analysed in this study.

EB Pruriginosa (Dominant)							
Patient no.	**Gender**	**Diagnosis**	***COL7A1* mutation**	**cDNA sequence change**	**Exon**	**MMP1 promoter GG SNP**	**Mutation reference**
1	M	EB-pr	p.Gly1522Glu*	c.4565 G>A	45	2G/1G	(Whittock *et al.*, 1999)
2	F	EB-pr	**p.Gly1770Asp**	c.5309 G>A	61	2G/2G	**This study**
3	F	EB-pr	p.Gly1791Glu	c.5372 G>A	62	2G/1G	(Mellerio *et al.*, 1999)
4	F	EB-pr	p.Gly1791Glu	c.5372 G>A	62	2G/1G	(Mellerio *et al.*, 1999)
5	M	EB-pr	p.Gly1791Glu	c.5372 G>A	62	2G/2G	(Mellerio *et al.*, 1999)
6	F	EB-pr	**p.Gly1860Arg**	c.5578 G>A	66	2G/1G	**This study**
7	F	EB-pr	**p.Gly1860Arg**	c.5578 G>A	66	2G/1G	**This study**
8	M	EB-pr	**p.Gly1913Arg**	c.5737 G>C	69	2G/1G	**This study**
9	F	EB-pr	p.Gly2040Asp	c.6119 G>A	73	1G/1G	(Whittock *et al.*, 1999)
10	F	EB-pr	p.Gly2043Arg*	c.6127 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
11	M	EB-pr	p.Gly2043Arg*	c.6127 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
12	F	EB-pr	p.Gly2043Arg*	c.6128 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
13	F	EB-pr	p.Gly2043Arg*	c.6129 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
14	F	EB-pr	**p.Gly2159Glu**	c.6476 G>A	79	1G/1G	**This study**
15	F	EB-pr	**p.Gly2159Glu**	c.6476 G>A	79	1G/1G	**This study**

16	F	EB-pr	**p.Gly2159Glu**	c.6477 G>A	79	1G/1G	**This study**
17	F	EB-pr	**p.Gly2213Arg**	c.6637 G>A,	83	2G/1G	**This study**
19	M	EB-pr	p.Gly2242Arg	c.6724 G>A	85	1G/1G	(Lee *et al.*, 1997)
20	M	EB-pr	p.Gly2251Glu	c.6752 G>A	86	2G/2G	(Shimizu *et al.*, 1999)
21	F	EB-pr	p.Gly2251Glu	c.6752 G>A	86	1G/1G	(Shimizu *et al.*, 1999)
22	F	EB-pr	p.Gly2251Glu	c.6752 G>A	86	2G/1G	(Shimizu *et al.*, 1999)
23	F	EB-pr	p.Gly2251Glu	c.6752 G>A	86	2G/1G	(Shimizu *et al.*, 1999)
24	F	EB-pr	p.Gly2251Glu	c.6752 G>A	86	2G/1G	(Shimizu *et al.*, 1999)
25	M	EB-pr	p.Gly2287Arg	c.6859 G>A	87	2G/2G	(Shimizu *et al.*, 1999)
26	M	EB-pr	p.Gly2287Arg	c.6859 G>A	87	2G/1G	(Shimizu *et al.*, 1999)
27	F	EB-pr	**p.Gly2290Ala**	c.6869 G>C	87	1G/1G	**This study**
28	F	EB-pr	p.Gly2366Val	c.7097 G>T	92	2G/2G	(Chuang *et al.*, 2004)
Non-EB Pruriginosa (Dominant)							
29	F	BDN	**p.Gly1483Asp**	c.4448 G>A	42	2G/2G	**This study**
30	M	BDN	p.Gly1522Glu*	c.4565 G>A	45	1G/1G	(Fassihi *et al.*, 2005)
31	M	BDN	p.Gly1522Glu*	c.4565 G>A	45	2G/1G	(Fassihi *et al.*, 2005)
32	M	DDEB	**p.Gly1773Asp**	c.5318G>A	61	2G/1G	**This study**
33	F	DDEB	**p.Gly2009Ala**	c.6026G>C	73	2G/2G	**This study**
34	M	DDEB	**p.Gly2028Glu**	c.6083 G>A	73	1G/1G	**This study**
35	F	DDEB	p.Gly2034Arg	c.6100 G>A	73	2G/2G	(Kon *et al.*, 1997b)

36	M	DDEB	p.Gly2034Arg	c.6100 G>A	73	2G/1G	(Kon *et al.*, 1997b)
37	M	DDEB	p.Gly2037Arg	c.6109 G>A	73	1G/1G	(Iwata *et al.*, 2006)
38	M	DDEB	p.Gly2037Glu	c.6110 G>A	73	2G/1G	(Jonkman *et al.*, 1999)
39	F	DDEB	p.Gly2043Arg*	c.6127 G>A	73	1G/1G	(Christiano *et al.*, 1995a)
40	F	DDEB	p.Gly2043Arg*	c.6127 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
41	M	DDEB	p.Gly2043Arg*	c.6127 G>A	73	2G/2G	(Christiano *et al.*, 1995a)
42	M	DDEB	p.Gly2043Arg*	c.6127 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
43	M	DDEB	p.Gly2043Arg*	c.6127 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
44	M	DDEB	p.Gly2043Arg*	c.6127 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
45	F	DDEB	p.Gly2043Arg*	c.6127 G>A	73	1G/1G	(Christiano *et al.*, 1995a)
46	M	DDEB	p.Gly2043Arg*	c.6127 G>A	73	2G/1G	(Christiano *et al.*, 1995a)
47	M	DDEB	**p.IVS 37+1 G>T**	c.IVS 37+1 G>T	73	1G/1G	**This study**
48	F	DDEB	p.Gly2055Glu	c.6164 G>A	73	2G/2G	(Christiano *et al.*, 1996)
49	F	DDEB	**p.Gly2067Ala**	c.6200 G>C	73	2G/2G	**This study**
50	F	BDN	**p.Gly2233Glu**	c.6698 G>A	73	2G/1G	**This study**
51	F	DDEB	p.Gly2239Asp	c.6716 G>A	85	2G/2G	(Tamai *et al.*, 1998)

Mutations in bold are novel, *mutations identified in this study with both EB-pr and non EB-pr phenotypes.DDEB-dominant dystrophic epidermolysis bullosa, EB-pr - epidermolysis bullosa pruriginosa, BDN- bullous dermolysis of the newborn, IVS, intervening sequence (intron); SNP-single nucleotide polymorphism

Table 6.2 The incidence of the 1G/2G SNP in the *MMP1* promoter area in dominant EB-pr, non EB-pr DDEB, RDEB and controls in this study.

	Dominant EB-pr n (%)	non EB-pr DDEB n (%)	RDEB n (%)	Controls n (%)
Total patient no.	n=27	n=23	n=25	n=50
Total allele no.	54	46	50	100
1 bg§1G/1G	7 (25.9)	6 (26.1)	2 (8.0)	16 (32.0)
2G/1G	15 (55.5)	10 (43.5)	15 (60.0)	26 (52.0)
2G/2G	5 (18.5)	7 (30.4)	8 (32.0)	8 (16.0)
Total 2G alleles/ Total 1G+2G alleles	25/54	24/46	31/50	42/100
GG allelic frequency	46.30%	52.20%	62%*	42%

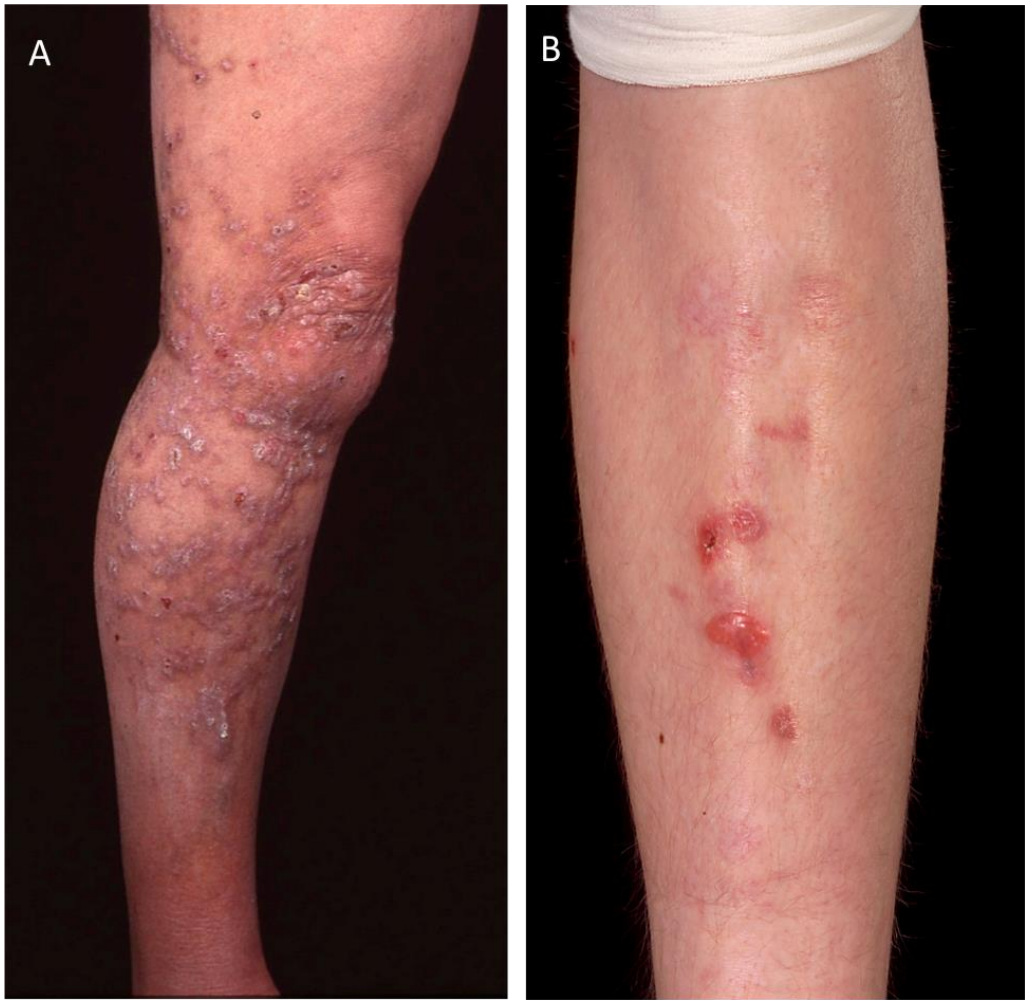
*p<0.02 compared to controls. DDEB-dominant dystrophic epidermolysis bullosa, EB-pr- epidermolysis bullosa pruriginosa, RDEB- recessive dystrophic epidermolysis bullosa.

In an attempt to explain the phenotypic heterogeneity encountered in subjects with the p.Gly2043Arg mutation in this study, the incidence of the 1G/2G alleles was analysed in 12 individuals with this mutation. 11 subjects expressed the 2G polymorphism on at least one allele, while one individual was homozygous for the 1G polymorphism and had non EB-pr dominant DEB phenotype. The 1G/2G allelic differences between the 2 clinical subgroups were not statistically significant.

Intra-familial variability was also noted in a previously reported pedigree affected by EB-pr (Ee *et al.*, 2007). Four family members from two generations demonstrated a variable clinical phenotype including BDN and EB-pr despite inheriting the same heterozygous GS mutation; p.Gly2251Glu. However, findings in this Chapter showed that there was no correlation between the presence or absence of the 2G allele and the EB-pr phenotype. Furthermore, a clinically unaffected 9-year-old offspring in the third generation was also found to harbour a heterozygous p.Gly2251Glu mutation, in addition to the 2G/1G SNP in the *MMP-1* promoter area. The inconsistency of the 2G SNP expression in this family probably excludes MMP-1 as a cause for the phenotypic heterogeneity. Other subjects with the heterozygous GS mutation p.Gly2043Arg had classical DDEB, EB-pr or BDN. However phenotypic heterogeneity could not be explained by the MMP-1 promoter 2G polymorphism alone (Figure 6.3).

In addition, the incidence of the 2G/1G SNP was studied in six subjects within the recessive DEB group known to have developed cutaneous squamous cell carcinoma before the age of 30 years. All were found to harbour the 2G variant on one or both *MMP1* alleles.

Figure 6.3 Phenotypic heterogeneity for the *COL7A1* glycine substitution mutation, p.Gly2043Arg. (A) p.Gly2043Arg heterozygosity results in EB-pr in a 65-year-old lady with prurigo-like lesions on the legs. (B) In contrast, the same mutation results in non EB-pr dominant DEB, with mild blistering and scarring on the shin in an 18 year-old male. Pruritus was not present in this young patient however, it is not possible to rule out development of a pruritic phenotype with advancing age.



6.4 Discussion

Molecular analysis in this study has identified 12 new heterozygous glycine substitutions mutations underlying EB-pr and non-pruritic (non EB-pr) dominant DEB expanding the current *COL7A1* glycine substitution mutation database to 133 mutations (71 dominant, 62 recessive). These new mutations were also incorporated into the novel mutation database outlined in Chapter 4. However, no significant differences were observed between the various subgroups when studying the nature or location of the EB-pr GS mutations compared to other dominant or recessive forms of DEB. Furthermore, analysis of the 1G/2G *MMP1* gene promoter polymorphism in this study showed that there is no association between the 2G allele and the EB-pr phenotype. Therefore it is unlikely that this particular polymorphism acts a genetic modifier for the pruritic EB-pr phenotype.

Nevertheless understanding the role of MMPs and TIMPs is potentially promising for future phenotype-genotype correlation in EB. As proteolytic enzymes, MMPs play a significant role in tissue remodelling, inflammation, healing and cancer invasiveness in DEB through degradation of the extracellular matrix (Ala-aho and Kahari, 2005; Chakraborti *et al.*, 2003; Ghilardi *et al.*, 2001; Ye *et al.*, 2001; Kivisaari *et al.*, 2008; Changotade *et al.*, 2007; Bodemer *et al.*, 2003). MMP7 facilitates cancer progression through cleavage of surface molecules such as E-cadherin and syndecan-1, as well as extracellular matrix proteins including fibrillin and collagen VII (Kivisaari *et al.*, 2008; Changotade *et al.*, 2007). Moreover, various MMPs were found to influence disease severity in RDEB as evidenced by varying clinical severity in 3 siblings affected with RDEB which was also shown to correlate with

increased levels of MMP2, MMP3 and MMP9 and reduced levels of TIMP1 (Bodemer *et al.*, 2003). In contrast, the study outlined in this Chapter did not identify a correlation between the *MMP-1* functional polymorphism and the various subtypes of dominant DEB. However, given the role of MMPs and TIMPs in various pathophysiological aspects of DEB, other genetic variants might be elucidated in the future as potential genetic modifiers in DEB. In addition, anti-proteolytic therapies might prove a useful clinical option particularly for recessive DEB, given the role of increased *MMP-1* expression and collagen VII degradation in some cases (Titeux *et al.*, 2008). Findings in this Chapter however, do not suggest that EB-pr subjects will specifically benefit from this form of treatment compared to other individuals with DEB.

In addition, the above results support the published data showing that the -1607 1G/2G polymorphic variant in the *MMP1* promoter may be associated with greater clinical severity in recessive DEB. It was also observed in this study that six subjects within the recessive DEB group developed cutaneous squamous cell carcinoma before the age of 30 years and each was found to harbour the 2G variant on one or both *MMP1* alleles. This observation is perhaps not surprising, given the role of MMPs in cancer invasion and progression (Chakraborti *et al.*, 2003; Ghilardi *et al.*, 2001; Ye *et al.*, 2001; Zinzindohoue *et al.*, 2005). In particular, the 2G *MMP1* polymorphism has been associated with an increased risk of developing colorectal, breast, ovarian, head and neck cancers where the expression levels of MMP-1 was found to correlate negatively with survival.

In conclusion, despite recent studies demonstrating a significant effect of the -1607 1G/2G polymorphic variant in the *MMP1* promoter on RDEB

phenotypic severity, the findings outlined in this Chapter did not demonstrate similar results in its dominantly inherited counterpart. This suggests that other genetic or environmental modifying factors play a role in the uniquely pruritic variant, EB-pr, and are yet to be identified. This would have important implications for future genetic counselling and genotype-phenotype correlation.

Chapter 7

Revertant Mosaicism in Recessive Dystrophic Epidermolysis Bullosa

Abstract

The natural correction of somatic mutations; revertant mosaicism (RM) has been described in several inherited disorders including JEB and EBS. The occurrence of this phenomenon in EB is likely to be more prevalent than anticipated, but at the time of thesis work there had been no reports of RM in DEB. This Chapter investigates the occurrence of non-fragile patches of skin in a 41-year-old patient with RDEB-sev gen as a possible consequence of RM. Genomic analysis of the peripheral leukocyte DNA revealed a compound heterozygote mutation; c.1732C>T (p.Arg578Stop) in exon 13 and c.7786delG (p.Gly2593fsX4) in exon 104. The same mutations were found in genomic DNA extracted from whole skin taken from both affected and unaffected skin. However, sequencing of cDNA from both areas revealed only a heterozygous nonsense mutation in unaffected skin compared to the compound heterozygous loss of function mutations found in blister-prone skin. Further analysis revealed nearly undetectable levels of collagen VII expression in unaffected skin fibroblasts indicating that keratinocytes are the site of mutation reversion and the source of functional collagen VII. Long-range sequencing was consistent with an intragenic crossover occurring somewhere between exons 21 and 104 resulting in a wild type allele and subsequent non blister-prone areas of skin. The detection of RM in RDEB offers an exciting model of 'natural cell therapy' with an unlimited resource for cell therapy and reflects a phenomenon that is probably overlooked in RDEB.

7.1 Introduction

Revertant mosaicism (RM) refers to the natural presence of two genetically distinct populations of cells as a result of spontaneous mutation correction during mitosis (Hall, 1988; Jonkman *et al.*, 1997). This naturally occurring phenomenon and through various potential mechanisms, results in restoration of wild type phenotype.

The RM phenomenon has been reported in several inherited diseases, including X-linked severe combined immunodeficiency (Stephan *et al.*, 1996), Bloom's syndrome (Ellis *et al.*, 1995), Fanconi's anaemia (Kalb *et al.*, 2007), X-linked Wiscott–Aldrich syndrome (Ariga *et al.*, 2001), Duchenne muscular dystrophy (Klein *et al.*, 1992), tyrosinemia type I (Demers *et al.*, 2003), Lesch-Nyhan adenosine deaminase deficiency (Hirschhorn *et al.*, 1994), Omenn syndrome (Wada *et al.*, 2005), X-linked ectodermal dysplasia (Nishikomori *et al.*, 2004), T-cell immunodeficiency (Rieux-Laucat *et al.*, 2006) and leucocyte adhesion deficiency (Tone *et al.*, 2007; Uzel *et al.*, 2008). It has also been increasingly described in genodermatoses including EB as well as ichthyosis en confetti (Choate *et al.*, 2010; Burger *et al.*, 2012) and dyskeratosis congenita (Vulliamy *et al.*, 1999; Jongmans *et al.*, 2012).

At the time this thesis began, with reference to EB, RM had only been reported in EBS and JEB as a result of *in vivo* reversion of mutations in *LAMB3, COL17A1* and *KRT14* (Darling *et al.*, 1999; Schuilenga-Hut *et al.*, 2002; Smith *et al.*, 2004; Pasmooij *et al.*, 2005; Pasmooij *et al.*, 2007; Jonkman and Pasmooij, 2009; Jonkman *et al.*, 1997) .

7.2 The incidence of revertant mosaicism

The reported incidence of RM generally, has been variable. It has been estimated to occur in up to 11% of patients with Wiskott-Aldrich syndrome (Stewart *et al.*, 2007), 18% of individuals with Fanconi's anaemia (Kalb *et al.*, 2007) and 23% of patients with Bloom syndrome (German *et al.*, 1977). Higher rates were reported in nHJEB (35%) (Jonkman and Pasmooij, 2009; Pasmooij *et al.*, 2012) and tyrosinemia type I (85%) (Demers *et al.*, 2003). However despite rare reports of RM, this phenomenon is thought to be more prevalent than expected, with many cases likely going unnoticed. One report by Pasmooij *et al.*, even suggested that *in vivo* reversion might occur in all patients with generalized nHJEB (Pasmooij *et al.*, 2012). In their Dutch cohort of 10 nHJEB patients, 6 patients with generalized disease showed genetic evidence of revertant mosaicism, where as the other four demonstrated clinical and photographic evidence of revertant skin patches. Also, in support of a high prevalence of RM is the fact that multiple *in vivo* correcting mutations can occur in the same individual as will be discussed later in this Chapter. In addition, revertant cells can be found in normal tissue as in the case of JEB where keratinocytes expressing collagen XVII were found in both clinically affected and unaffected skin, adding to the hypothesis that many cases of revertant mosaicism are undetected (Pasmooij *et al.*, 2005).

7.3 Mechanisms of revertant mosaicism

Various corrective mechanisms have been observed in RM, including back mutations, second-site mutations, intragenic crossovers and mitotic gene

conversions (Jonkman *et al.*, 1997; Pasmooij *et al.*, 2005; Frank and Happle, 2007; Kvittingen *et al.*, 1994; Ellis *et al.*, 1995; Jonkman and Pasmooij, 2009; Jonkman *et al.*, 2003; Chen *et al.*, 2007). Back mutation refers to the restoration of wild type sequence, whereas second-site mutations involve additional exonic or intronic compensatory mutations that lead to the restoration of the open reading frame. Intragenic cross-over results from the exchange of genetic material between two alleles whereas in mitotic gene conversion one allele is altered by genetic material received from a donor allele that remains unchanged. Different corrective phenomena have also been observed in the same patient (Pasmooij *et al.*, 2005; Jonkman and Pasmooij, 2009). Another rare mechanism for RM is that of DNA slippage, which occurs during replication, particularly in genes with a high GC content. This was observed in 3 patients with Wiskott-Aldrich syndrome from the same family in whom a known 6 base pair insertion mutation was deleted as a result of proposed DNA slippage resulting in restoration of wild type sequence (Wada *et al.*, 2001; Wada *et al.*, 2004). Another mechanism observed in mammals, is that involving transposons also known as the 'jumping genes'. These are sequences of genetic material that can alter their position leading to induction or reversion of mutations (McClintock, 1956; Druker and Whitelaw, 2004; Whitelaw and Martin, 2001). Although transposons have been observed in Duchenne muscular dystrophy their role in RM is yet to be verified (Bittar and Happle, 2005; Pizzuti *et al.*, 1992).

7.3.1 Revertant mosaicism: a random or directed process?

It is still unclear whether the occurrence of RM represents a directed corrective process or merely a random natural phenomenon. Some hypotheses in support of the former, suggest that the presence of an ancestral RNA-sequence cache could lead to a template-directed mutation reversion (Lolle *et al.*, 2005). However, there are abundant proposals in support of a random corrective process. In particular is the observation that the average rate of human mutagenesis is around 2.5×10^{8} per nucleotide per generation with *in vivo* reversion estimated at 0.39 per cell division (Nachman and Crowell, 2000). This not-so-rare-process might also be influenced by several potential modulators including innate DNA instability, mutagenic metabolites, UV radiation, as well as feto-maternal factors (Choate *et al.*, 2010; Demers *et al.*, 2003; Pasmooij *et al.*, 2012; Bergeron *et al.*, 2004). In tyrosinemia type I, the high rate of RM, estimated to occur in 85% of cases, is thought to result from the intracellular accumulation of mutagenic metabolites fumarylacetoacetate and maleylacetoacetate as a consequence of fumarylacetoacetate hydrolase deficiency (Demers *et al.*, 2003). In ichthyosis en confetti the abnormal localisation of keratin 10 within the nucleus is thought to lead to an increased recombination mutation rate and silencing of the mutant allele resulting in the characteristic widespread confetti-like revertant patches (Choate *et al.*, 2010). On the other hand, in DNA instability syndromes such as Fanconi's anaemia and Bloom syndrome, the increased mutation rate is thought to contribute to the high occurrence of RM (Lai-Cheong *et al.*, 2011). Moreover, UV radiation, through an increase in mutagenesis has also been implicated in RM, although this has not been validated (Pasmooij *et al.*, 2012). It has also been proposed

that feto-maternal cell trafficking could explain the occurrence of two genetically distinct population of cells in RM cases, however studies in tyrosinemia type I, did not support this hypothesis (Bergeron *et al.*, 2004).

7.3.2 Revertant mosaicism: a single or multiple event?

The occurrence of multiple corrective somatic reversions affecting different cell lineages in the same patient also weakens the argument for a directed corrective process in RM (Pasmooij *et al.*, 2007). Multiple revertant clones have been observed in tyrosinemia type I (Bliksrud *et al.*, 2005), Omenn syndrome (Wada *et al.*, 2005), severe combined immunodeficiency disease (Rieux-Laucat *et al.*, 2006), Wiskott–Aldrich syndrome (Boztug *et al.*, 2008), as well as JEB (Pasmooij *et al.*, 2007; Pasmooij *et al.*, 2005). Differing somatic reversions were also shown to involve B-lymphocytes, T-lymphocytes as well as natural killer cells in 2 siblings with Wiskott-Aldrich syndrome (Boztug 2008). Although the mechanisms by which these multiple events occur are still unclear, the potential mutagenic modulators outlined above could contribute to an increased mutation rate, as do potential mutational hot spots such as direct repeats or homonucleotide tracts (Hamanoue *et al.*, 2006).

Moreover, the multiple reversion mutations can occur by differing mechanisms as demonstrated in one cohort of 14 patients with nHJEB (Pasmooij *et al.*, 2012). In this study, the *COL17A1* mutation, c.2237delG, was shown to be corrected by five different *in vivo* reversion events in the same patient with no predilection for a particular correction mechanism (Pasmooij *et al.*, 2012). This was also observed in Wiskott–Aldrich Syndrome where over

30 different corrective mutations were observed within the same patient (Davis *et al.*, 2010a). This is in contrast to ichthyosis en confetti, an autosomal dominant disease, where only mitotic combination revertant mutations are observed (Choate *et al.*, 2010). It is possible though, that other unidentified mutations with differing reversion mechanisms are yet to be discovered.

7.4 The clinical effects of revertant mosaicism

The implications of RM on phenotype are dependent on various factors including the timing of reversion and its relation to stem cells, the mechanism of genetic correction and whether it leads to complete restoration of wild type sequence, as well as the extent of selective advantage of revertant cells.

The precise timing of RM phenomena is still unclear. However, the expanding nature of many revertant patches noted in EB, suggests that this phenomenon occurs earlier on during embryogenesis to affect regenerative stem cells (Davis and Candotti, 2010b). It might also explain why RM is a common phenomenon in inherited conditions affecting self-regenerating organs such as skin, liver and blood. The timing of RM events during embryogenesis is also reflected by the type of reverted cells, such as hepatocytes, lymphocytes, muscle cells and fibroblasts (of mesodermal origin), as well as keratinocytes (of ectodermal origin). Involvement of the latter for example, would suggest reversion after the formation of the 3-layered embryo and following the completion of blashko lines, owing to the clinical appearance and distribution of the revertant patches (Pasmooij *et al.*, 2012; Pasmooij *et al.*, 2007; Pasmooij *et al.*, 2005).

The mechanism of RM could also affect the degree of phenotypic amelioration. Reversions resulting in complete restoration of wild type such as back mutations result in normal protein expression and wild-type phenotype. However, mechanisms such as second-site mutation might not result in a completely normal protein owing to an abnormal intervening genetic sequence and therefore, although reversion might result in clinical amelioration the wild-type phenotype is not fully restored (Lai-Cheong *et al.*, 2011; Pasmooij *et al.*, 2005). Furthermore, clinical improvement may not be seen in cases of partial RM. This phenomenon has been described in nHJEB and EBS secondary to mutations in *COL17A1* and *KRT14* respectively. In one case, a deletion mutation in *COL17A1* was partially corrected leading to the expression of an immunohistochemically reactive, albeit non-functional, protein. In another example, an EBS patient homozygous for the splice site mutation c.526-2A>C showed immunohistochemical expression of K14 in some areas, yet no reversion was detected on genomic DNA. However, mRNA analysis revealed an additional transcript, which led to the expression of a semifunctional protein that was detected immunohistochemically and ultrastructurally but that did not result in any clinical amelioration.

In addition, revertant cells have been associated with selective growth advantage *in vivo*, which is reflected by the occurrence of large expanding patches of revertant skin in some cases. This could also be a reflection of the degree of stem cell reversion or possibly the effects of wild-type protein on cell function (Pasmooij *et al.*, 2005; Hamanoue *et al.*, 2006; Davis *et al.*, 2010a; Stewart *et al.*, 2007; Stephan *et al.*, 1996; Wada *et al.*, 2003; Jonkman and Pasmooij, 2012).

In this Chapter, an individual with RDEB found to have 2 areas of non-fragile skin is studied. Somatic revertant mosaicism is explored as a potential explanation for this unique clinical finding.

7.5 Case study

A 41-year-old Caucasian British man was diagnosed with RDEB-sev gen resulting in mutilating scars with bilateral mitten deformities, nutritional deficiencies, growth retardation, oesophageal strictures and recurrent squamous cell carcinomas. Despite generalised skin fragility, two small patches of skin on his left wrist and right shin never blistered despite repeated trauma. Examination of these two non-fragile areas, that measured approximately 85 cm^2, revealed skin of normal appearance and texture (Figure 7.1).

To explain this phenotypic heterogeneity, and following ethics committee approval (St Thomas' Hospital Ethics Committee: 07/H0802/104) and informed consent and in accordance with the Declaration of Helsinki principles. Blood samples were taken from the patient, his brother and parents for mutational analysis. The skin from the left wrist was also investigated with biopsies that I took from both the blister-prone (unreverted) and the normal-appearing (reverted) areas and I performed collagen VII immunolabelling (Figure 7.2), as well as the reverse transcriptase PCR and mutational analysis including long range sequencing as described in Chapter 2. TEM was performed by Patricia Dopping-Heppenstal at the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory. The area of potential RM on the patient's right shin was not investigated in this study.

Figure 7.1 Clinical assessment of two clinically revertant areas of skin. In a 40-year-old male patient with severe RDEB, two patches of skin on the left wrist and right shin showed no evidence of skin fragility. These were clinically and texturally similar to normal skin.

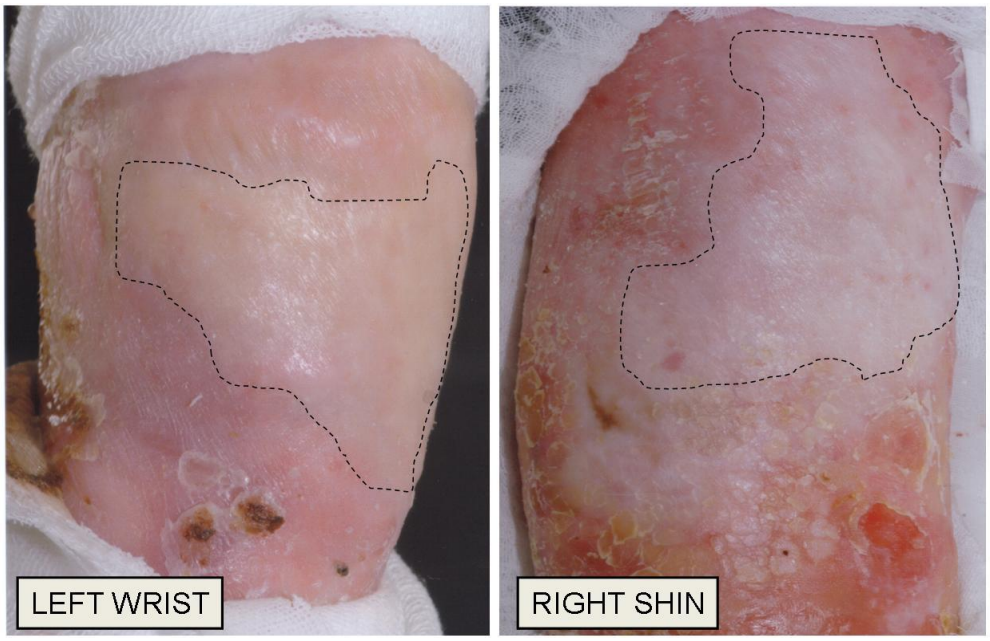
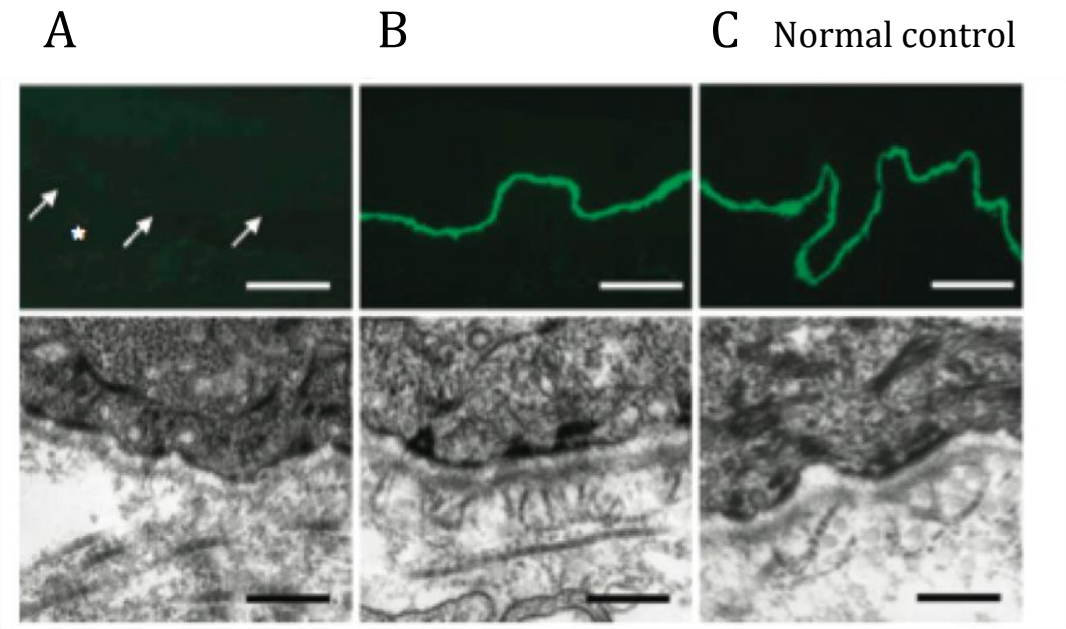


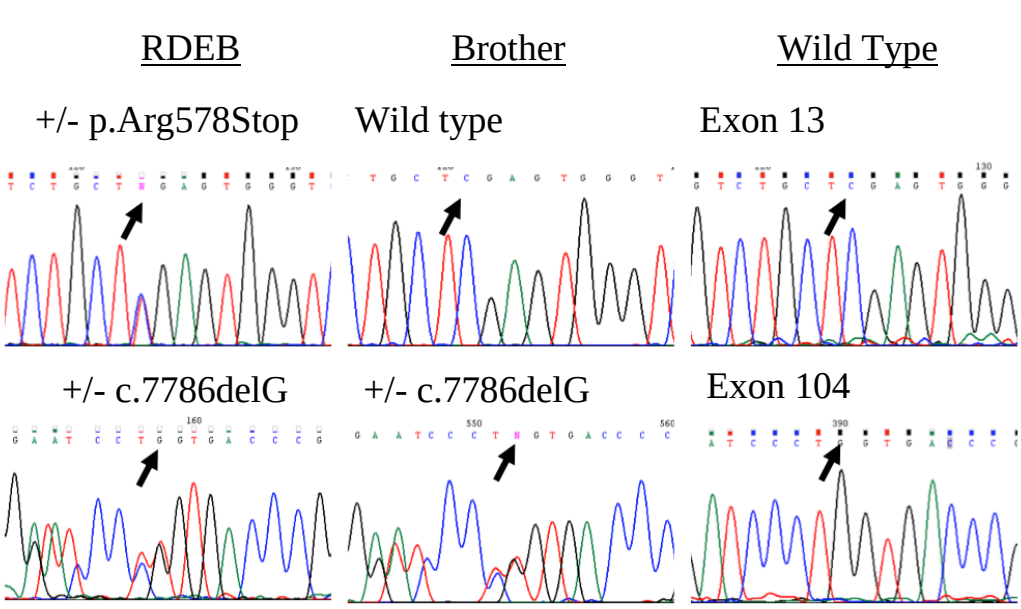
Figure 7.2 Immunohistochemical and ultrastructural findings in reverted, unreverted and normal skin. Immunohistochemical analysis revealed complete absence of collagen VII expression at the basement membrane in unreverted skin with absence of anchoring fibrils and blister formation on ultrastructural level (arrows depict DEJ, asterisk indicates a blister) (A). This is in contrast to reverted skin, which shows bright linear collagen VII labelling at the basement membrane comparable to that of normal skin, as well as demonstrable anchoring fibrils (B). However, these seem shorter and less discernable than in normal skin (C). Bars for immunofluorescence microscopy images 50μm; bars for transmission electron microscopy images 0.1μm.



7.6 Results and discussion

Genomic analysis of the proband's peripheral leukocyte DNA revealed a compound heterozygote mutation involving two *COL7A1* loss-of-function mutations; c.1732C>T (p.Arg578Stop) in exon 13 (maternal) and c.7786delG (p.Gly2593fsX4) in exon 104 (paternal) (Figure 7.3). Both of these mutations were shown to be recurrent within the white British population (Mellerio et al., 1997). The brother was a carrier for the p.Arg578Stop mutation.

Figure 7.3 Peripheral blood genomic DNA analysis. Mutation analysis revealed a compound heterozygous mutation in the affected proband. His brother was a carrier of the deletion mutation c.7786delG in exon 104.



To explain the phenotypic heterogeneity genomic DNA was extracted from whole skin taken from both reverted and unreverted skin, which revealed similar findings to those observed in peripheral genomic DNA (Figure 7.4A). To explore the phenotypic heterogeneity further, genomic RNA extraction and reverse transcriptase-PCR across the sites of both mutations was performed. This was done to identify any genotypic heterogeneity on a transcriptional level. Sequencing of cDNA from both reverted and unreverted areas revealed only a heterozygous nonsense mutation in reverted skin compared to the compound heterozygous loss of function mutations found in unreverted skin (Figure 7.4B). To explore the discordance between the whole skin gDNA and cDNA analysis, RNA was extracted from fibroblasts in both affected and unaffected areas. Real time RT-PCR revealed that the collagen VII expression in revertant whole skin was similar to that observed in the brother who was a heterozygous carrier. However the level of expression in fibroblasts harvested from revertant skin was nearly undetectable (Figure 7.5). This indicates that keratinocytes are the site of mutation reversion and the source of functional collagen VII. The latter was reflected by the presence of anchoring fibrils on TEM and positive BM labelling on immunofluorescence. The presence of both unreverted fibroblast and reverted keratinocyte mixture in whole skin samples could explain the inability to detect reversion on whole skin gDNA analysis.

Figure 7.4 *COL7A1* mutational analysis in reverted, unreverted and normal skin. (**A**) Sequencing of gDNA from unreverted skin reveals compound heterozygosity for the mutations c.1732C>T (p.Arg.578Stop) in exon 13 and c.7786delG (p.Gly2593fsX4) in exon 104. Both mutations are present in gDNA from the reverted skin compared to wild type. (**B**) Sequencing of cDNA extracted from unreverted skin shows the presence of both mutations. In contrast, in the patient's reverted skin, only the nonsense mutation p.Arg.578Stop can be identified.

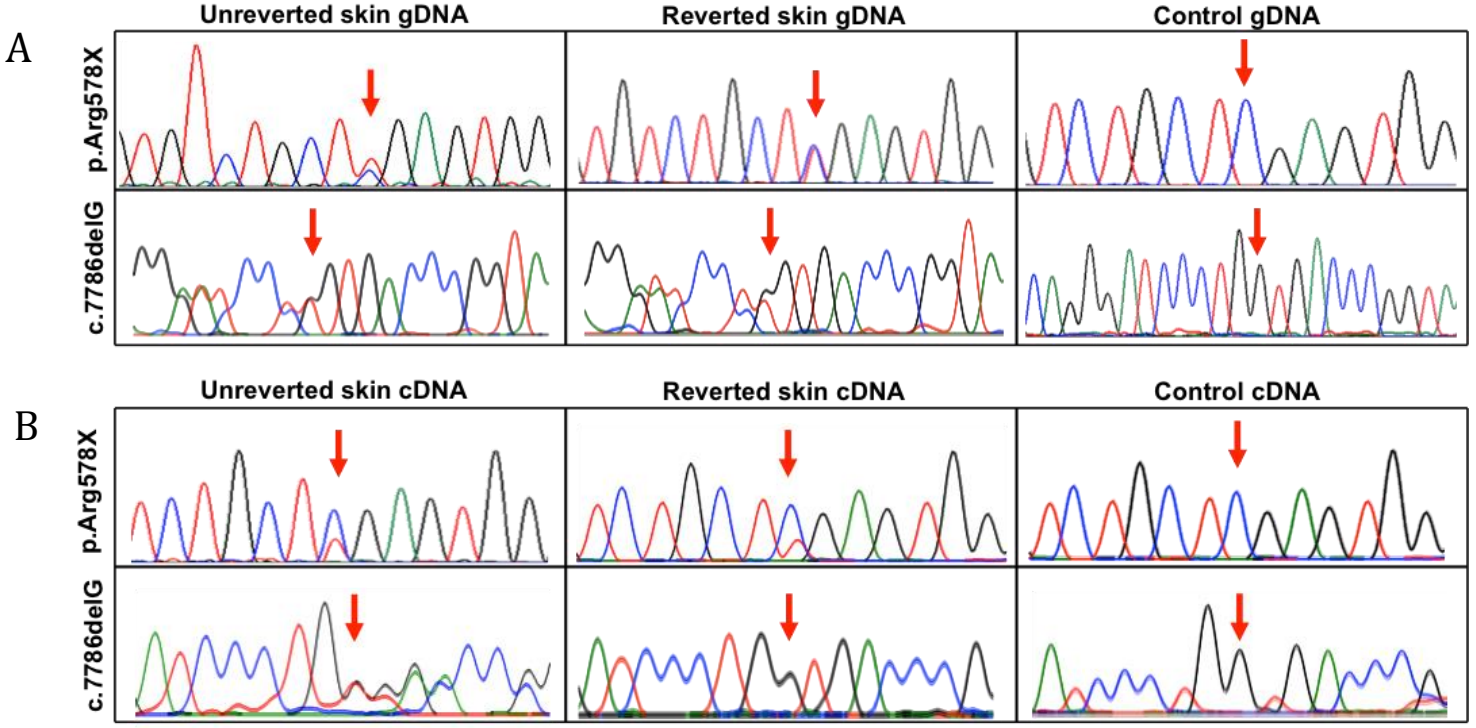
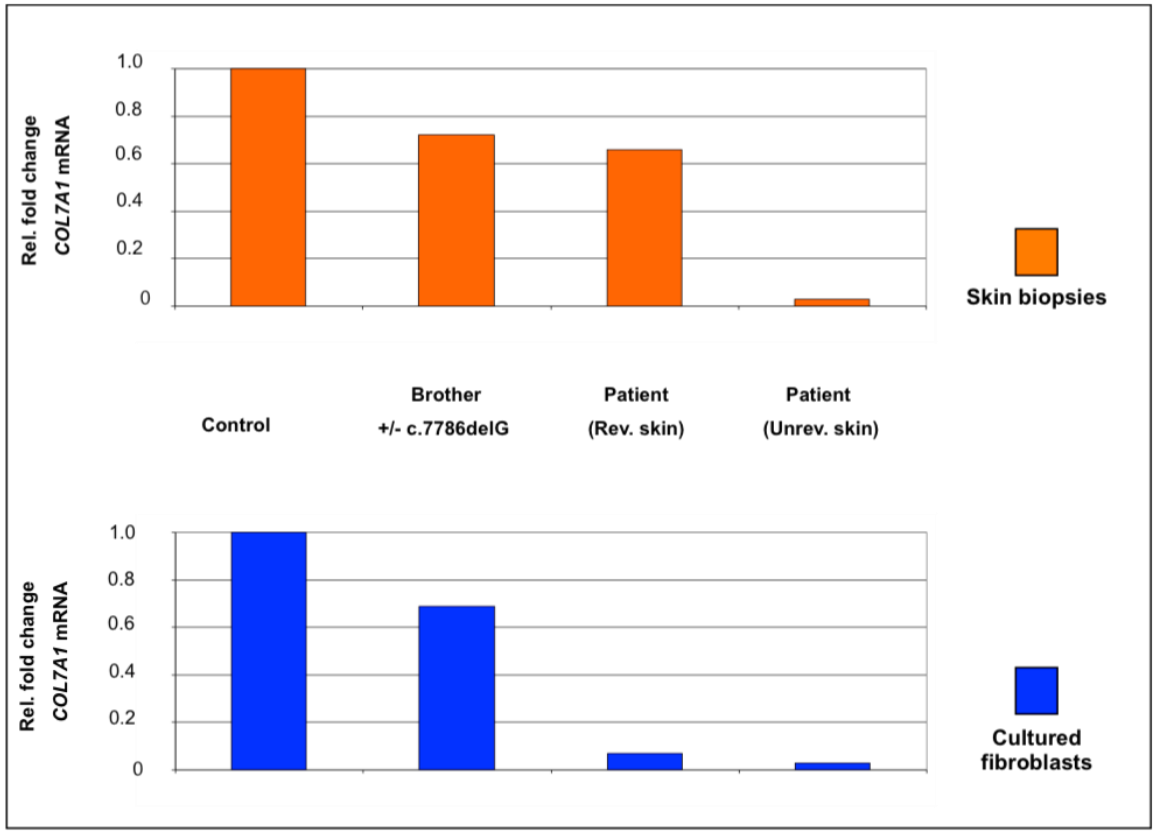
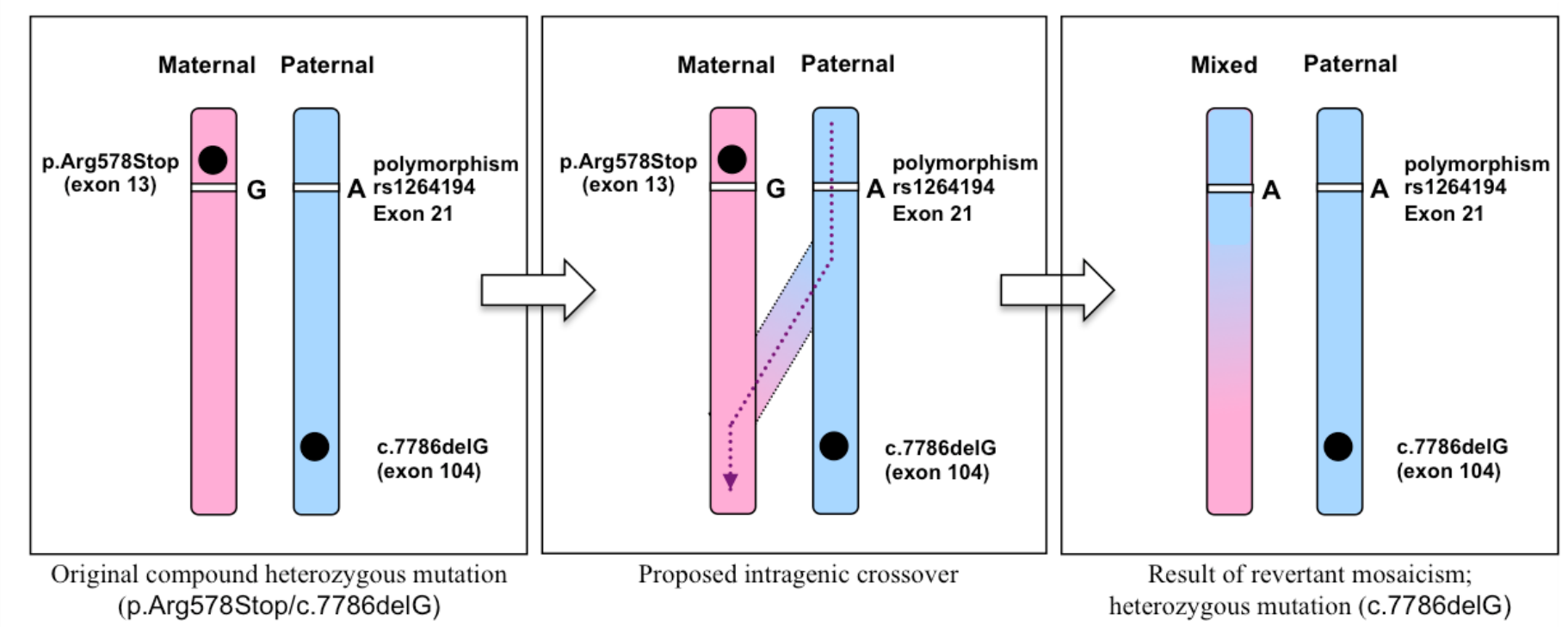


Figure 7.5 *COL7A1* expression in whole skin and fibroblasts. Real-time RT-PCR shows barely detectable *COL7A1* expression in affected skin or cultured fibroblasts derived from unreverted areas. In contrast, reverted skin shows *COL7A1* expression levels similar to those in the patient's brother (a heterozygous carrier). However, cultured fibroblasts from the reverted area fail to show a similar increase in *COL7A1* gene expression.



To explore the mechanism of loss of compound heterozygosity, long-range sequencing of the patient's reverted skin cDNA was performed using LongAmp Taq DNA polymerase as described in Chapter 2. In addition, polymorphism analysis was performed to distinguish between maternal and paternal alleles. This identified differences for a common *Pvu*II polymorphism in exon 21 (c.2817G/A; rs1264194 A allele- paternal, G –allele- maternal). The heterozygosity was present at the level of cDNA and gDNA in both the affected as well as the unaffected skin. However, sequencing revealed a *COL7A1* allele bearing neither the c.1732C>T (p.Arg578Stop) in exon 13 (maternal) nor the c.7786delG (p.Gly2593fsX4) in exon 104 (paternal). This was detected in combination with a paternally derived A nucleotide for the exon 21 polymorphism. Collectively, this data is consistent with an intragenic crossover occurring somewhere between exons 21 and 104 resulting in a wild type allele (Figure 7.6). This results in the expression of collagen VII which is reflected clinically by the presence of a patch of skin that is functionally similar to that present in the proband's brother who is a heterozygous carrier for the loss-of-function mutation p.Arg578Stop.

Figure 7.6 A schematic outlining the proposed intragenic crossover. The maternal nonsense mutation in exon 13 occurs on the same allele bearing a G nucleotide for a *Pvu*II polymorphism in exon 21, but in the reverted skin the wild-type sequence for exon 104 occurs on the same allele bearing a paternal A nucleotide for the exon 21 polymorphism. This suggests that intragenic crossover has occurred somewhere between exons 21 and 104, and that the patient has restored one functional *COL7A1* allele.



The occurrence of RM in genodermatoses in particular, provides an exciting model for studying RM, as the occurrence and behaviour of revertant patches, in addition to effects of therapy can be easier to identify clinically. In RDEB, the novel identification of a RM patch of skin that shows functional properties similar to those of a heterozygous carrier is likely to provides new therapeutic opportunities for patients. This potentially unlimited resource of 'natural cell therapy' can be utilized for skin directed therapy such as skin grafts or even for the induction of pluripotent stem cells (iPSCs) capable of differentiating into both haematopoietic and mesenchymal stem cells.

With regards to skin directed therapy, Gostynski *et al.* used adhesive stripping to remove the epidermis from revertant skin patches in a patient with nHJEB (Gostynski *et al.* 2009). This was followed by the culture of keratinocytes to create skin grafts that were later transplanted onto affected skin. However, the level of revertant keratinocytes in the biopsies and subsequently the grafts was insufficient to achieve full functional repair. This could be explained by the low level of mosaicism in culture, estimated at 30% of revertant keratinocytes. This reduced to only 3% in transplanted grafts (Gostynski *et al.*, 2009). It is also likely to reflect a low number of revertant stem cells within the graft, an observation that was explored in another study by Mavilio *et al.* (Mavilio *et al.*, 2006). Multiple biopsies of revertant patches were taken from a patient with nHJEB for gene therapy. Holoclone-forming cells were only identified in 2 palmar biopsies but were undetectable in other biopsies from most of the patient's skin. This is thought to be due to the continuous proliferative stimulus induced by chronic wound healing observed in nHJEB. The palmar biopsies were then subjected to retroviral *LAMB3*

genetic correction followed by culture of corrected keratinocytes, which were used for subsequent grafting. This resulted in full functional correction that was sustained on a molecular and clinical level for over 5 years with no reported long-term side effects (Mavilio *et al.*, 2006; De Luca *et al.*, 2009).

One potential method of increasing the yield of epidermal stem cells in grafts would be the use of punch grafting which utilizes full thickness skin obtained by punch biopsies for the purpose of wound healing (Nordstrom and Hansson, 2008; Thami *et al.*, 2004; Kirsner *et al.*, 1996; Yuen *et al.*, 2013). This has only been applied in RM recently, where revertant skin was punch grafted into chronic ulcers in patients with nHJEB. Complete ulcer re-epithelialization occurred within 14 days of grafting with no subsequent loss of skin integrity during the 18 months follow-up period (Gostynski *et al.*, 2014).

However, a more definitive way of generating an unlimited resource of gene corrected cells is that by inducing iPSCs from revertant colonies. This is an exciting emerging technique by which patient-specific iPSCs can be used for skin directed cell therapy or even bone marrow transplantation. Gene corrected iPSCs have already been generated from patients with recessive dystrophic EB, which were capable of differentiating to both hematopoietic and non-hematopoietic lineages including keratinocytes (Tolar *et al.*, 2011; Tolar *et al.*, 2014; Itoh *et al.*, 2011). In addition, iPSCs were shown to differentiate into both hematopoietic and mesenchymal stem cells capable of homing into blistered skin following bone marrow transplantation (Tolar *et al.*, 2011; Wagner *et al.*, 2010). iPSCs generated from reverted colonies has the potential of providing an unlimited patient specific resource of corrected cells

obviating the need for induced genetic correction.

In conclusion, this Chapter outlines the first description of RM in an individual with RDEB. In the case studied, RM is likely a result of intragenic cross-over in the *COL7A1* gene, which resulted in the generation of wild type alleles and the restoration of collagen VII expression at the basement membrane with detectable anchoring fibrils in reverted skin. The RM phenomenon is likely to be more common than initially thought as evidenced by the publication of 3 subsequent cases of RDEB and Kindler syndrome with proven RM (Pasmooij *et al.*, 2010; van den Akker *et al.*, 2012; Lai-Cheong *et al.*, 2012). This represents an exciting resource for patient directed cell therapy and gives further insight into the RM phenomenon in heritable skin diseases.

Chapter 8

Cell therapy: The role of intradermal allogeneic fibroblasts on collagen VII expression in RDEB

Abstract

Intradermally injected allogeneic fibroblasts in individuals with RDEB survive for only a few days but can increase collagen VII at the DEJ for several weeks or months. The precise mechanism and duration of the response, however, are not known. A more detailed analysis was undertaken in one RDEB individual and found that a single fibroblast injection led to increased collagen VII labelling at the DEJ for ~9 months (returning to baseline levels by 12 months) and increased *COL7A1* expression for ~3 months (back to baseline by 6 months). The elevated *COL7A1* expression was paralleled by increased expression of *HB-EGF*, encoding heparin-binding EGF-like growth factor. Treatment of control and RDEB cultured keratinocytes and fibroblasts with recombinant HB-EGF led to increased *COL7A1* gene expression, with increased AP-1 transcription factor levels. Injection of fibroblasts into the margins of chronic wounds in the RDEB subject led to rapid re-epithelialization. The collective data indicate that HB-EGF may be a key cytokine in the response of RDEB keratinocytes to allogeneic fibroblast injections in increasing mutant but partially functional collagen VII at the DEJ, and that a single injection of cells can improve wound healing and alter the RDEB intrinsic skin protein defect for several months.

8.1 Introduction

Significant advances have been made in the last decade with regards to cell therapy in DEB as discussed in Chapter 1. Understanding of the cellular, molecular and ultrastructural pathology underlying DEB has paved the way for the delivery of cell based therapy utilising keratinocytes, fibroblasts, stem cells and bone marrow derived progenitors to increase collagen VII expression at the BMZ. Moreover, progress has been made in the mode of delivery of cell therapy to involve cutaneous applications, systemic infusion as well as bone marrow transplantation (Table 8.1).

8.2 Skin directed cell-therapy

Local cell therapy in the form of allografts (McGrath *et al.*, 1993b; Falabella *et al.*, 2000; Falabella *et al.*, 1999), *ex-vivo* genetically corrected keratinocyte sheets or organotypic skin cultures (Woodley 2003, Ortiz-Urda 2002) have been utilised to restore skin integrity and to treat intractable skin ulcers in RDEB. However, this mode of localised cell therapy is limited by variable success of engraftment, infection, fragility of keratinocyte sheets, difficult gene transfer into epidermal keratinocyte and biosafety issues, as well as, low level of maintained collagen VII expression (Sat *et al.*, 2000; Ghazizadeh *et al.*, 1999). Similarly, allogeneic dermal substitutes did not alter collagen VII expression as evidenced by immunohistochemical and ultrastructural studies (Kubo and Kuroyanagi, 2004; Natsuga *et al.*, 2010; Hasegawa *et al.*, 2004).

Table 8.1 An outline of the evolution of cell therapy in RDEB at the time of this study.

Study Reference	Cell Source	Recipient	Method	Outcome
Skin Directed Topical Cell Therapy				
McGrath *et al.*, 1993b	Allogeneic normal human keratinocytes	Human adult RDEB	*In vivo* grafting	Clinically there was little improvement in wound healing. Transient expression of some collagen VII epitopes was noted but not anchoring fibrils.
Verplancke *et al.*, 1997	Autologous split-thickness graft from unaffected skin covered by allogeneic normal human keratinocytes	Human paediatric RDEB	*In vivo* grafting	Clinically there was stable improvement in wound healing for 3 months but collagen VII expression was not assessed.
Eisenberg *et al.*, 1998	Allogeneic human keratinocyte and fibroblast skin equivalents	Human paediatric RDEB	*In vivo* grafting	Clinically there was accelerated wound healing with normal anchoring fibrils on TEM.
Falabella *et al.*, 2000	Allogeneic human keratinocyte and fibroblast skin equivalent (Apligraf)	Human paediatric RDEB and JEB	*In vivo* grafting	Clinically there was accelerated wound healing but collagen VII expression was not assessed.
Wollina *et al.*, 2001	Autologous keratinocytes on an esterified hyaluronic acid membrane	Human adult RDEB	*In vivo* grafting	Clinically there was stable improvement in wound healing for over 12 months but collagen VII expression was not assessed.

Fivenson *et al.*, 2003	Allogeneic human keratinocyte and fibroblast skin equivalent (Apligraf)	Human paediatric RDEB and JEB	*In vivo* grafting	Clinically there was accelerated wound healing but collagen VII expression was not assessed.
Hasegawa *et al.*, 2004	Allogeneic cultured dermal substitute	Human RDEB	Topical application to intractable wound surface	Clinically there was accelerated wound healing but collagen VII expression was not assessed.
Natsuga *et al.*, 2010	Allogeneic cultured dermal substitute	Human adult RDEB	Topical application to intractable wound surface	Clinically there was accelerated wound healing although there was no demonstrable increase in collagen VII expression.
Chen *et al.*, 2002b	Gene-corrected human DEB keratinocytes and fibroblasts	Severe combined immunodeficiency mice	*In vivo* grafting	There was increased expression of collagen VII at the DEJ with formation of anchoring fibrils in human grafts.
Goto *et al.*, 2006	Gene-corrected human DEB keratinocytes and fibroblasts	Nude athymic rat	Transplantation of gene-corrected DEB keratinocyte and fibroblast grafts into rat wounds	Expression of collagen VII in the DEJ of the transplanted grafts was higher using gene-corrected RDEB fibroblasts compared to gene-corrected RDEB keratinocytes.
Bilousova *et al.*, 2011	Undifferentiated mouse iPSCs or iPSC-keratinocytes were mixed with dermal fibroblasts	Nude athymic mouse	*In vivo* grafting	This study showed that iPSCs could differentiate into keratinocytes capable of regenerating a fully differentiated epidermis *in vivo*.

Skin Directed Injectable Cell Therapy				
Ortiz-Urda *et al*., 2003	*Human RDEB fibroblasts overexpressing collagen VII, RDEB fibroblasts and normal fibroblasts*	Nude athymic mouse	Single intradermal injection into intact mouse skin and human RDEB organotypic skin-equivalents	*COL7A1* gene-corrected fibroblasts produced stable collagen VII and anchoring fibril expression *in vivo* for the length of the study (16 weeks).
Woodley *et al.*, 2003	*Human RDEB fibroblasts overexpressing collagen VII, RDEB fibroblasts and normal fibroblasts*	Nude athymic mouse	Single intradermal injection into intact mouse skin	*COL7A1* gene-corrected fibroblasts produced stable collagen VII and anchoring fibril expression in vivo for 4 months. Also normal human fibroblasts increased collagen VII expression at the DEJ as effectively as gene-corrected fibroblasts when injected at a density of 5x106 cells.
Fritsch *et al.*, 2008	Mouse wild-type and hypomorph RDEB fibroblasts	Immunocompetent collagen VII hypomorphic mouse (expressing 10% of normal collagen VII levels)	2 Intradermal injections 7 days apart	Stable expression of collagen VII at the DEJ was noted with improved skin integrity for at least 100 days.
Kern *et al.*, 2009b	Mouse wild-type fibroblasts, hypomorph RDEB fibroblasts or normal human fibroblasts	Immunocompetent collagen VII hypomorphic mouse (expressing 10% of normal collagen VII levels)	2 Intradermal injections 7 days apart	Stable expression of collagen VII at the DEJ was noted with improved skin integrity for at least 100 days but no significant side effects or immune response to collagen VII.

Wong *et al.*, 2008	Autologous and allogeneic human fibroblasts	Human adult RDEB	Single intradermal injection	An increase in collagen VII expression and anchoring fibril formation was maintained at 3 months mainly due to increase in the patient's own collagen VII with subsequent formation of rudimentary anchoring fibrils. None of the 5 patients developed collagen VII antibodies.
Conget *et al.*, 2010	Human allogeneic msc	Human adult RDEB	Single Intradermal injection	There was evidence of increased collagen VII expression at the DEJ at 4 months with accelerated skin healing.
Liao *et al.*, 2013	Human cord blood-derived unrestricted somatic stem cells	Immunocompromised mouse	Single intradermal injection 1cm from the wound edge or intravenous injection	There was evidence of migration of USSCs into wound from a distant intradermal injection site as well as following systemic injection with subsequent accelerated healing.
Systemic Cell Therapy				
Woodley *et al.*, 2007	Human RDEB fibroblasts overexpressing collagen VII and normal fibroblasts	Nude athymic mouse	Intravenous	*COL7A1* gene-corrected fibroblasts homed to mouse skin wounds with accelerated skin healing. There was increased expression of collagen VII at the DEJ with formation of anchoring fibrils that was sustained for the duration of the study (8 weeks).
Tolar *et al.*, 2009	Wild type mouse bone marrow cells	*Col7a1*-/- mouse	BM infusion	Proof of principle study showing that wild type BM cells homed to wounded skin with increased collagen VII expression and anchoring fibril formation at the DEJ as well as improved overall survival.

Chino *et al.*, 2008	CD90 depleted mouse bone marrow cells	*Col7a1*-/- mouse	BM infusion	BM cells homed to skin even without prior injury with increased collagen VII expression and fibrillar structure formation at the DEJ, as well as improved overall survival.
Wagner *et al.*, 2010	Whole human bone marrow from matched related donor	Human paediatric RDEB	Intravenous infusion with myeloablative conditioning	All patients demonstrated skin and marrow chimerism and clinical improvement. 5 out of 6 recipients showed increased levels of collagen VII at the DEJ.
Tolar *et al.*, 2011	Whole bone marrow from siblings, unrelated donor and unrelated cord blood donor and allogeneic MSCs	Human paediatric RDEB and JEB	Intravenous infusion	4 out of 12 patients died due to complications or disease progression. There was clinical improvement in all assessed patients and increased collagen VII expression in 3 RDEB patients. There was increased laminin-332 expression at the DEJ in 1 JEB patient.

BM; bone marrow, DEJ; dermoepidermal junction, iPSCs; induced pluripotent stem cells, JEB; junctional epidermolysis bullosa, MSCs; mesenchymal stem cells, RDEB; recessive dystrophic epidermolysis bullosa, USSCs; unrestricted somatic stem cells.

Therefore, to enhance delivery and increase bioavailability of cells *in vivo*, intradermal injection of keratinocytes and fibroblasts was initially attempted in animal models. In one study, regeneration of normal skin was demonstrated *in vivo* by infusion of lentivirus transduced DEB keratinocytes and fibroblasts into the skin of severe combined immunodeficiency (SCID) mice (Chen *et al.*, 2002b). This was followed by other studies where gene-corrected fibroblasts were intradermally injected into RDEB organotypic skin-equivalents, which have been grafted onto immunodeficient mice. In both animal models, intradermal cell injection resulted in restoration of collagen VII expression at the DEJ (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003).

Both keratinocytes and fibroblasts were used for cell therapy up until this point. However, although keratinocytes were shown to produce more collagen VII *in vivo*, studies in murine models revealed that gene-corrected RDEB fibroblasts resulted in greater collagen VII expression at the DEJ compared to gene-corrected RDEB keratinocytes (Goto *et al.*, 2006). Therefore, these findings coupled to the optimization in fibroblast delivery, paved the way for intradermal allogeneic fibroblast injections in RDEB patients and will be discussed in more details later in this Chapter (Wong *et al.*, 2008; Yan and Murrell, 2010). Moreover, understanding of stem cell biology and the role of BM derived progenitors on wound healing, has led to the intradermal infusion of allogeneic BM derived mesenchymal stromal cells (MSCs) in RDEB patients (Conget *et al.*, 2010). In a study by Conget *et al.*, a single injection resulted in increased collagen VII expression at the DEJ and improved healing of chronic ulcers that lasted for 4

months. In addition, *in vivo* studies have demonstrated that local and systemic injection of human umbilical cord blood-derived unrestricted somatic stem cells (USSCs) was found to promote wound healing and skin regeneration in mouse models. The USSCs even if injected intradermally at a distant site were found to be capable of migrating to areas of wounding (Liao *et al.*, 2013).

8.3 Systemic delivery of cell therapy

Further attempts to increase the bioavailability of cell therapy led to the intravenous injection of human fibroblasts into transgenic RDEB mice (Woodley *et al.*, 2007). In this study, fibroblasts were shown to home to skin, leading to increased collagen VII expression at the BMZ and promoting wound healing. Similarly, preclinical studies in *COL7A1* null animal models demonstrated that the systemic infusion of bone marrow derived stem cells resulted in amelioration of collagen VII expression and subsequent reduction of skin fragility. Survival was also extended in the treated mouse embryos (Tolar *et al.*, 2009; Chino *et al.*, 2008).

More recently, *in vivo* studies have demonstrated that local and systemic injection of human umbilical cord blood-derived USSCs was found to promote wound healing and skin regeneration in immunocompromised mouse models with full-thickness excisional wounds (Liao *et al.*, 2013). In this study, it was shown that USSCs specifically migrate to wounds even following distant intradermal or systemic injection of USSCs.

These studies paved the way for clinical trials using bone marrow transplantation in a murine model of RDEB (Tolar *et al.*, 2009) and subsequently the infusion of matched allogeneic bone marrow stem cells with chemoablative pre-conditioning in children with RDEB (Wagner *et al.*, 2010). This led to partial correction of collagen VII expression with amelioration of skin fragility. However, this procedure is fraught with risks relating to immunosuppression, carcinogenesis and incomplete correction. Therefore, measures under consideration to reduce morbidity and mortality include use of reduced intensity chemoablation and co-infusion of MSCs (Uitto *et al.*, 2012; Petrova *et al.*, 2010; Conget *et al.*, 2010). Other promising alternatives include the infusion of homologous transgenic stem cells or patient specific-RDEB iPSCs cells in an attempt to achieve safer patient specific therapy (Tolar *et al.*, 2011; Murauer *et al.*, 2011; Uitto, 2011b). RDEB iPSCs derived from fibroblasts and keratinocytes were found to differentiate into both mesenchymal and haematopoietic stem cells and produced collagen VII *in vivo* (Tolar *et al.*, 2011; Uitto, 2011b; Bilousova *et al.*, 2011). A significant draw back with the use of iPSCs however, relates to the increased risk of malignancy owing to the use of transcription factors that could potentially lead to oncogenic effects (Liang *et al.*, 2012).

8.4 The evolution of fibroblasts cell therapy

Although initial studies involving allogeneic skin grafts, keratinocytes sheets as well as skin bio-equivalents offered novel therapeutic options they were faced with multiple limitations (Eisenberg and Llewelyn, 1998; Fivenson *et al.*,

2003; McGrath *et al.*, 1993b; Verplancke *et al.*, 1997; Wollina *et al.*, 2001). These include the high risk of graft loss, which was estimated at 40% in transplanted burn wounds (Woodley *et al.*, 1988; Gallico *et al.*, 1984). In addition grafting is associated with technical limitations, pain, infection, scarring and requires intensive wound care. Therefore the emergence of fibroblast cell therapy offered a long awaited novel therapeutic option that was relatively safe and less invasive than skin grafting.

The application of fibroblast cell therapy in RDEB was first attempted in animal models by 2 independent studies (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003). Ortiz-Urda *et al.* demonstrated that intradermal injection of *COL7A1* gene corrected human RDEB fibroblasts overexpressing collagen VII directly into immune deficient mouse skin as well as regenerated RDEB human skin led to localisation and restoration of collagen VII expression at the BMZ for up to 4 months (Ortiz-Urda *et al.*, 2003). Woodley *et al.* achieved similar results in both *in vitro* and *in vivo* animal studies but also noted that normal fibroblast can secrete and deposit collagen VII at the BMZ as effectively as gene corrected fibroblasts, a finding that could be related to the higher number of fibroblasts used in this study (5×10^6 for intradermal injections as opposed to 1×10^6 fibroblasts in the previous study) and possibly earlier passage cells (Woodley *et al.*, 2003). The same group later explored the intradermal injection of self -inactivating lentiviral vector expressing human collagen VII (Woodley *et al.*, 2004b) as well as recombinant collagen VII into mouse skin (Woodley *et al.*, 2004a). The latter confirmed that there is a dose dependent expression of collagen VII in the skin either by

increasing the amount of recombinant collagen VII injected or the fibroblast density.

The relevance of fibroblast cell therapy on cell healing was demonstrated in a later study by Woodley *et al*. in which they showed that normal human fibroblasts or genetically corrected RDEB fibroblasts over-expressing collagen VII injected intradermally into athymic mice homed to wounded skin leading to an increase in collagen VII at the BMZ as well as anchoring fibril formation. Significantly, this was associated with significant enhancement of wound healing at multiple sites (Woodley *et al.*, 2007).

This encouraging data in animal models paved the way for intradermal injection of allogeneic fibroblasts in humans. In a study by Wong *et al.* a single intradermal injection of 5 $x10^6$ allogeneic human fibroblasts in non-blistered RDEB skin was found to increase collagen VII deposition at the DEJ and anchoring fibril formation, for up to three months (Wong *et al.*, 2008). This was reflected by reduced skin fragility at a clinical level. The type-VII collagen at the DEJ was shown to increase at 2 weeks and at 3 months following injection, which was associated with an increase in anchoring fibrils, however, none of these had normal morphology. No clinical or immunopathologic side effects were noted. The sustained increase in collagen VII expression was despite the fact that allogeneic fibroblasts where shown to be absent at 2 weeks. In the 5 RDEB patients enrolled in this study, the ones with mild reduction of collagen VII at baseline demonstrated higher and more sustained collagen VII expression following

allogeneic fibroblast injection raising question about the source of collagen VII production and pathways involved in expression upregulation.

8.5 Intradermal allogeneic fibroblast therapy: how does it work?

Multiple studies have suggested that the increase in collagen VII expression noted following allogeneic fibroblast injection is due to an increase in the patient's own mutant collagen VII production rather then wild type collagen secreted by the allogeneic fibroblasts. This is supported by findings by Wong *et al.* of an increase in structurally defective anchoring fibrils following intradermal fibroblast injections (Wong *et al.*, 2008). In addition, the increased expression was despite the fact the allogeneic fibroblast were no longer viable by 2 weeks. This led to the suggestion that allogeneic fibroblasts, and through a paracrine effect on keratinocytes and possibly fibroblast, could lead to increased synthesis of mutant yet partially functional collagen VII. This might explain the observation that patients with the highest baseline expression of collagen VII showed better response to allogeneic fibroblast injections as opposed to those subjects with little or no baseline expression. However, the increased expression at 2 weeks and 3 months observed in this study was not coupled by a matched cytokine gene expression. The study also observed intraepidermal collagen VII in patients receiving allogeneic but not autologous fibroblasts emphasizing yet again that at least a proportion of newly generated collagen VII is mutant protein.

A dose related effect corresponding to either the cells injected or the

protein generated was debated. In support of this is the finding in mouse models that increasing the amount of fibroblast cell number or amount of recombinant collagen VII leads to increased expression of collagen VII (Woodley *et al.*, 2003; Woodley *et al.*, 2004b; Woodley *et al.*, 2004a; Ortiz-Urda *et al.*, 2003). This was also supported in human studies where RDEB patients showed a lower expression of collage VII expression following parent carrier fibroblast injections compared to unrelated donor fibroblast. This might be a reflection of a lower collagen VII expression known to occur in carriers of *COL7A1* mutations (Wong *et al.*, 2008).

8.6 Limitations of fibroblast cell therapy

Although intradermal delivery of fibroblasts presents a less invasive form of therapy, it is not a definitive form of therapy and is associated with side effects including significant pain at the multiple sites of injection. It would also require a vast number of cells to cover large treatment areas. Genetically modified cells have the potential of tumourigenesis and allogeneic fibroblast injections could potentially lead to antibody formation and rejection. However, the latter was not observed in studies involving allogeneic fibroblast therapy (Wong *et al.*, 2008; Griffiths *et al.*, 2004; Falanga *et al.*, 1998; Woodley *et al.*, 2007; Price *et al.*, 2004). This could be explained by several factors including the short life span of allogeneic fibroblasts found to be as short as 1 and 2 weeks in animal and human studies respectively (Price *et al.*, 2004; Wong *et al.*, 2008). In addition, fibroblasts have a low immunological impact as they lose their cell surface HLA proteins in culture (Theobald *et al.*, 1993), a process that also leads to the loss of professional

antigen presenting cells such as the Langerhans cells (Phillips *et al.*, 2002), in addition to their ability in reducing T cell proliferation (Haniffa *et al.*, 2007).

8.7 Case study

For this study a 24-year-old Caucasian female with RDEB-sev gen was selected. She had been one of five subjects studied by our group in the first human intradermal fibroblast cell therapy study by Wong *et al.* (case 5; Wong *et al.*, 2008). This patient is known to be compound heterozygous for the *COL7A1* nonsense mutation c.2044C>T (p.Arg682Stop) in exon 15 of the NC1 domain and the splice site mutation IVS87+4A>G in intron 87. This particular donor splice-site mutation creates a leaky splice site, which leads to either in-frame skipping of exon 87 (69-bp) or wild-type sequence allowing some expression of full-length collagen VII (Whittock *et al.*, 1999). This also allows for tracking of the mutant allele in skin biopsy complementary DNA.

Of particular interest is that this patient showed higher levels of collagen VII expression at the BMZ at baseline compared to the other subjects in the study with only a slightly reduced collagen VII immunolabelling at the DEJ and anchoring fibril-like structures on TEM. She was subsequently shown to develop a higher collagen VII expression following allogeneic fibroblast injection, which was sustained for 3 months. To investigate this further, cutaneous allogeneic fibroblasts injections were performed in this patient but clinical, immunohistochemical and ultrastructural follow up was conducted for a total of 12

months.

8.8 Materials and Methods

For this study, I identified a 10 x 10cm area on the subject's upper back that was suitable for allogeneic fibroblast injections (non-blistered). At day 0, the fibroblasts or saline were injected intradermally into the mid-to- superficial dermis after applying topical anaesthesia with lidocaine/prilocaine cream (EMLA^{TM}) for 30minutes. The injections were performed by the Principal Investigator Prof. John McGrath using an insulin syringe. Each injection contained 5 x 10^6 neonatal allogeneic fibroblasts in 0.25ml of buffer sufficient to cover a 1cm^2 area. A similar volume of normal saline was also injected to adjacent skin for comparison following observations by Venugopal *et al.* that saline can also increase collagen VII in RDEB skin (Venugopal *et al.*, 2010) and took biopsies at days 15 and 90 from these saline injected areas .

Unlike the allogeneic fibroblasts used by Wong *et al*., the cell product (ICX-RHY- 013) used in this study, was supplied in pre-packed vials by Intercytex Ltd, Manchester, UK. The suspension consisted of allogeneic human dermal fibroblasts in Hypothermosol®-FRS in a sterile solution. The human dermal fibroblasts were isolated from neonatal foreskin from donors whose mothers had been screened for a range of diseases. The fibroblasts were cryopreserved, thawed and expanded in culture under good manufacturing practice (GMP) principles. Each vial contained 20 x 10^6 cells/ml and the dose used was 0.25ml of 20 x10^6

cells/ml (ICX-RHY- 013) per 1cm^2.

Skin biopsies (6mm punch biopsies) were taken at days 7, 15, 30, 90, 180, 270, and 360. I took all biopsies following informed consent and under aseptic techniques as described in Chapter 2. Tissue was then subdivided for immunofluorescence microscopy, transmission electron microscopy, storage in RNA later for microarray analysis and quantification of collagen VII gene expression (identical to protocols described in the Wong *et al.* study, Wong *et al.*, 2008). The end point of the study was 12 months after the initial injections.

The study was approved by the Guy's and St Thomas' Hospital local Ethical Committee (REC reference 04/Q0702/121) and was conducted according to the Declaration of Helsinki Principles. I made the amendments to the ethics application that was supplied by Dr. Wong for the study described above (Wong *et al.*, 2008), outlining the aims of the study, supporting evidence, the revised protocol and consent form. This was submitted to the local ethics committee and subsequently approved.

I performed Immunofluorescence studies, quantification of collagen VII fluorescence, RNA extraction from skin biopsies, cDNA synthesis, *in vitro* HB-EGF studies and *COL7A1* gene expression by quantitative real-time RT-PCR were performed as described in Chapter 2. The latter was carried out using *COL7A1* TaqMan® Gene Expression Assays (*COL7A1* Hs01574745_g1, Applied Biosystems; Foster City, CA, USA) which measured the amplification of the exon 54-55 fragment of the *COL7A1* cDNA. The exon skipped allele of the investigated

RDEB patient was separately amplified and quantified using qRT-PCR with a SYBR Green protocol (Applied Biosystems) using the following primers: *COL7A1* cDNA exon 86 forward 5`-GTGCCAGTGGAAAAGATGGA-3`, *COL7A1* cDNA exon 87 forward 5`- CGGACCTAAAGGAGAACCTG-3` and *COL7A1* cDNA exon 90 reverse 5`-AGTCCTCGGTCACCTTTGG-3`.

Gene expression microarray experiments were performed using total RNA extracted from baseline (day 0), fibroblast injected sites at days 7, 15 and 90, and the saline injected site at day 15. Whole genome gene expression microarray in each extracted RNA sample was performed using Sentrix Human-6 Whole Genome Expression Beadchips (Illumina Inc, San Diego, CA) as described in Chapter 2. The microarray data were confirmed with validated Taqman assays (FOS Hs00170630_m1, *JUN* Hs01103582_s1, *IL1R2* Hs01030385_m1, *LAMC2* Hs01043707_m1, *GAL* Hs01032385_m1, *STAT1* Hs01014002_m1, *TNFSF13B* Hs00198106_m1, *IRF1* Hs00971960_m1, *VCAM1* Hs01003372_m1, *COL1A1* Hs01076756_g1, *COL4A1* Hs01007434_g1, *COL7A1*, *COL17A1* Hs00166711_m1, *CCL18* Hs00268113_m1, *HB-EGF* Hs00181813_m1) purchased from Applied Biosystems (Foster City, CA, USA) using qRT-PCR).

8.9 Results

Following allogeneic fibroblast injection collagen VII expression at the BMZ increased after 15 days and was maintained for at least 270 days returning to baseline levels by 360 days. Using the saline control, a slight increase in collagen VII labelling was noted at day 15, but this returned to baseline by 90 days as

shown in Figure 8.1 and Table 8.2.

Using quantitative RT-PCR, *COL7A1* gene expression following fibroblast injection, showed a >20-fold increase at days 15 and 90, but this returned to baseline at day 180 (Figure 8.2A). Saline negative control injections resulted in an approximately 5-fold increase in *COL7A1* gene expression at day 15, but were similar to baseline levels at day 90. Using primers spanning the splice-site mutation in intron 87, the ratio of wild-type to mutant transcripts, at baseline, was approximately 2:3 (Figure 8.2B), which persisted in all biopsy material following allogeneic fibroblast injections.

Using whole genome microarray analysis, no differences were noted in gene expression for cytokines already known to increase *COL7A1* expression (supplementary electronic material). However, a >3-fold increase in expression of the gene for heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Iwamoto and Mekada, 2000) was noted. Quantitative real-time RT-PCR showed a similar temporal pattern to the *COL7A1* quantitative real-time RT- PCR data (linear correlation, $r = 0.978$; $P<0.0001$; Figure 8.2C). It was also noted that gene expression profiles of FOS (linear correlation, $r = 0.864$; $P<0.0006$) and JUN (linear correlation, $r = 0.945$; $P<0.0001$) were also highly similar to the pattern of increased *COL7A1* expression at the different time points (figure 8.3). JUN and FOS form the AP-1 transcription complex, which can bind to the *COL7A1* promoter and enhance gene expression (Nakano *et al.*, 2001)

Figure 8.1. Immunofluorescence labelling of collagen VII at the DEJ at baseline and following allogeneic fibroblast injection as well as saline control. Values of mean fluorescence intensities are also shown. Bars = 50 μm.

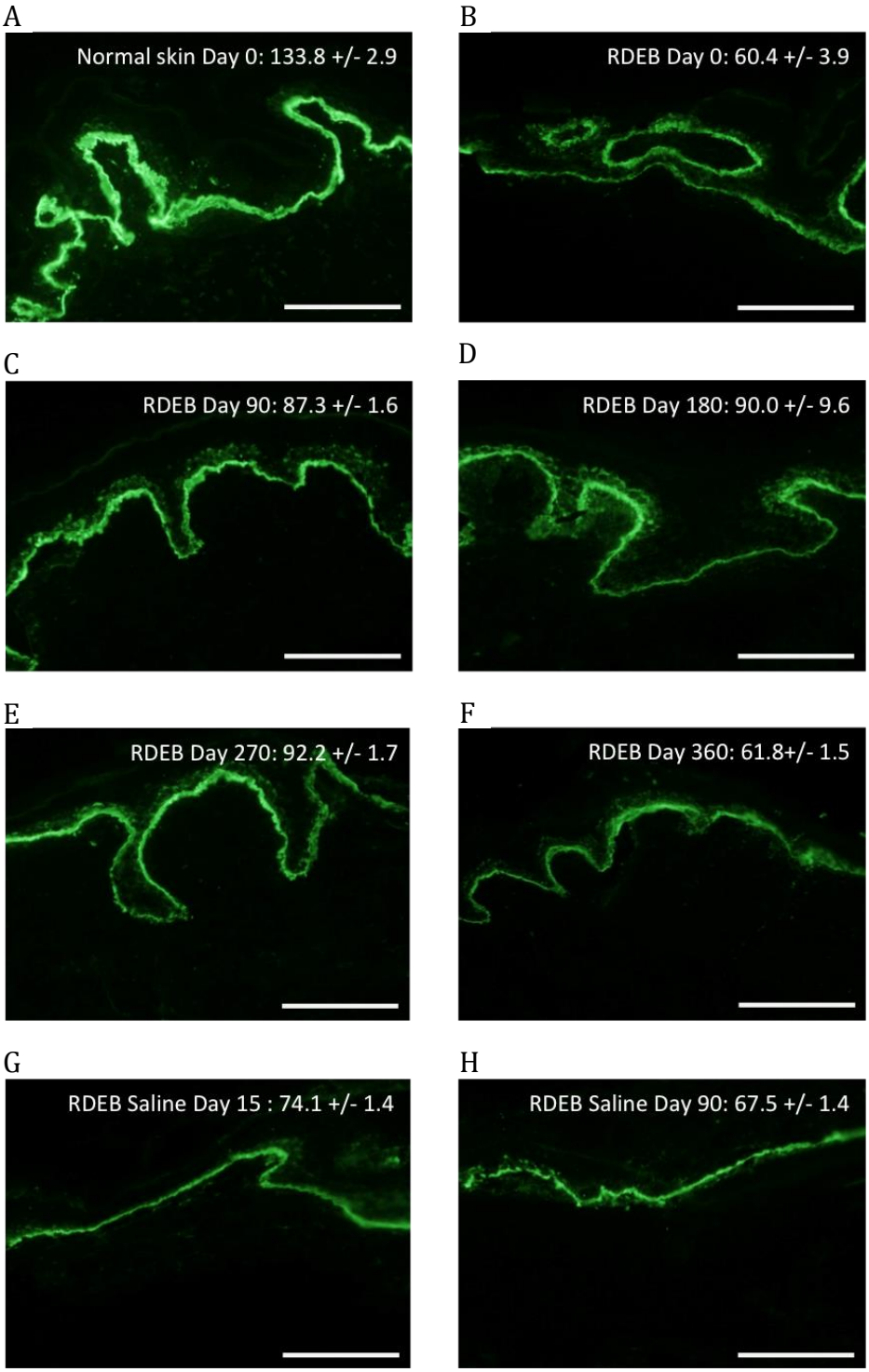
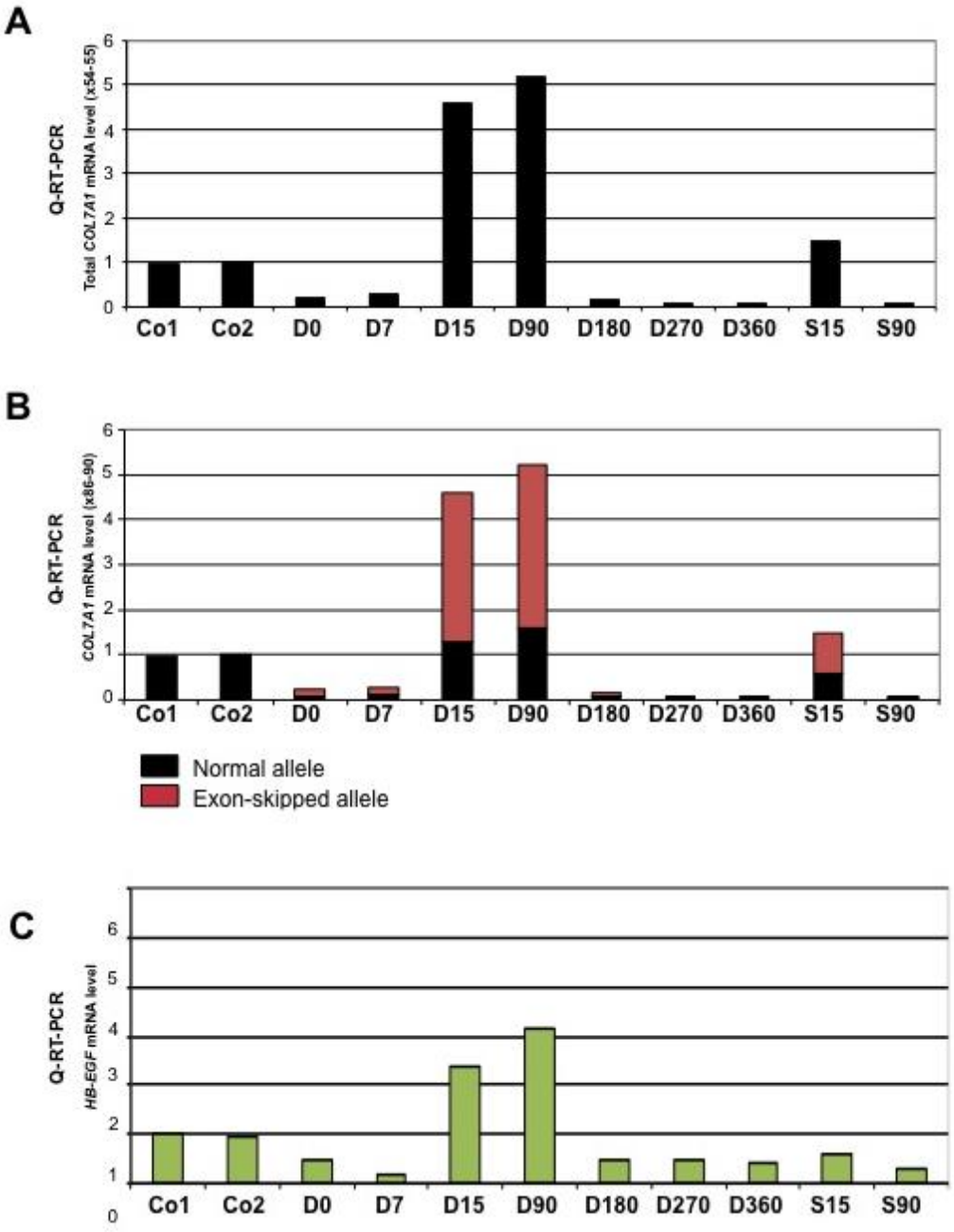


Table 8.2. Quantification of mean immunofluorescence intensity reveals increased collagen VII expression at the DEJ following allogeneic fibroblast injection and following intradermal saline injection to a lesser degree. D refers to day after fibroblast injection; S refers to days after saline injection.

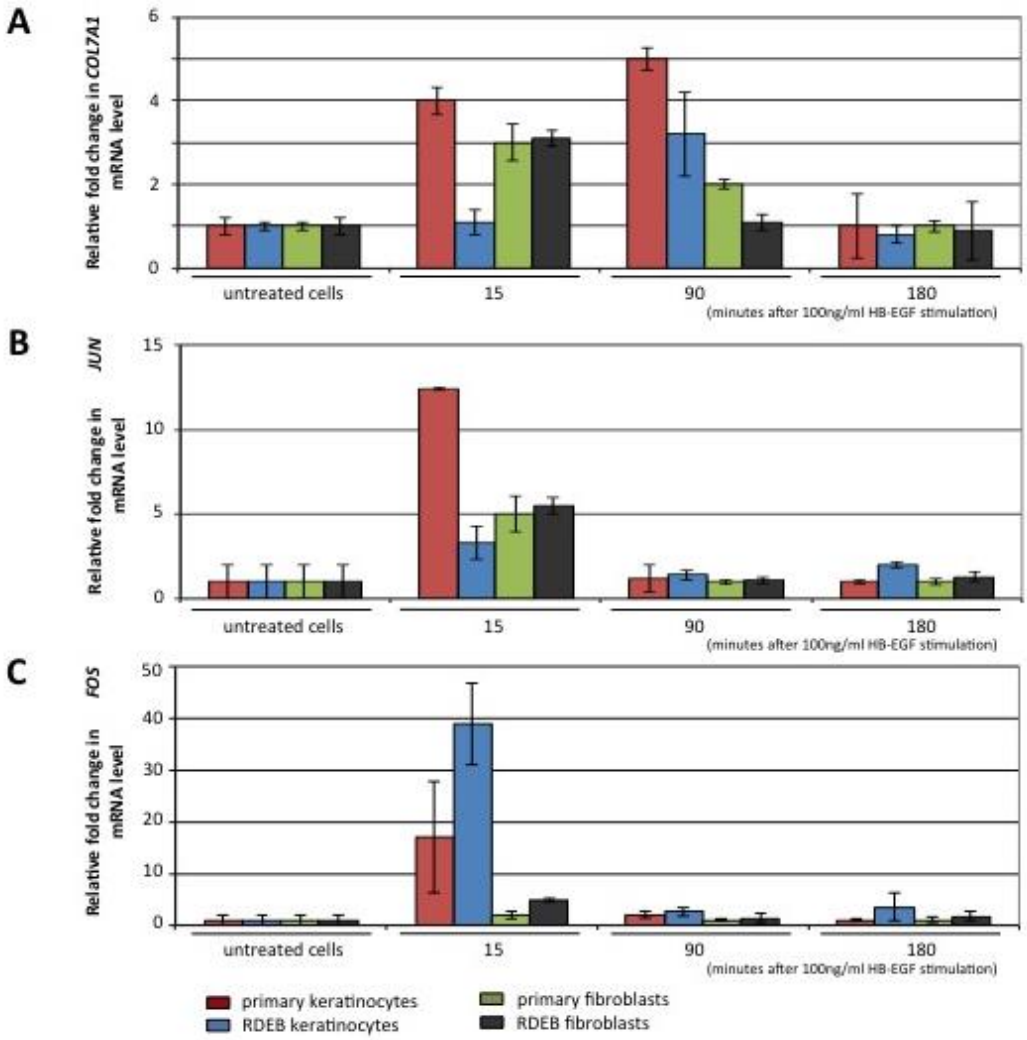
Skin type	Day	Mean IF intensity +/- SE
Normal Human Skin		133.8 +/- 2.9
RDEB	D0	60.4 +/- 3.9
RDEB	D7	87.3 +/- 1.6
RDEB	D180	90.0 +/- 9.6
RDEB	D270	92.2 +/- 1.7
RDEB	D360	61.8 +/- 1.5
RDEB	S15	74.1 +/- 1.4
RDEB	S90	67.5 +/- 1.4

Figure 8.2. Quantitative real-time RT-PCR (qRT-PCR) studies. There is increased expression of *COL7A1* (A,B) and heparin-binding epidermal growth factor-like growth factor (*HB-EGF*) (C) for at least 90 days following a single injection of allogeneic fibroblasts and a smaller, more transient increase following saline injection.



To investigate whether there is a temporal relationship between the upregulation of HB-EGF and increased *COL7A1* expression, HB-EGF studies were performed using subconfluent cultured keratinocytes and fibroblasts taken from normal controls and from two subjects with RDEB (including the subject of this study and an unrelated patient with the *COL7A1* mutations c.1732C>T (p.Arg578Stop) and c.7786delG (p.Gly2593fsX4). HB-EGF treatment led to upregulation of *COL7A1* mRNA in keratinocytes and fibroblasts in all cells (Figure 8.3A), as did treatment with transforming growth factor-β1. There was also an increase in *JUN* and *FOS* expression levels in all cells (Figure 8.3B and C).

Figure 8.3. Relative fold change in *COL7A1*, *JUN* and *FOS* mRNA levels following keratinocytes and fibroblasts treatment with recombinant heparin-binding epidermal growth factor-like growth factor (HB-EGF). (A) *COL7A1* expression; (B) *JUN* expression; (C) *FOS* expression.



8.10 Discussion

This study analysed the effects of intradermal allogeneic fibroblast injection for up to 360 days and demonstrated that a single injection capable of increasing collagen VII labelling at the DEJ almost by 1.5 fold and this was maintained for >9 months. *COL7A1* gene expression was upregulated for >3 months and was mirrored by an increase in the expression of *HB-EGF*. *In vitro*, the incubation of keratinocytes and fibroblasts with HB-EGF was shown to lead to increased *COL7A1* expression, potentially via increased AP-1 transcription factor. Saline controls injections were also associated with a mild but short-lived increase in collagen VII. The hypothesis for this is that allogeneic fibroblasts (and to a much lesser extent saline) induce subclinical inflammation that leads to the upregulation of *HB-EGF*. This upregulation is potentially mediated by the recipients' own keratinocytes and may be sustained for several months in an autocrine manner (Hashimoto *et al.*, 1994; Goishi *et al.*, 1995). The study also shows that HB-EGF can increase the *COL7A1* gene expression in cultured normal or RDEB keratinocytes and fibroblasts, supporting that HB-EGF might mediate the increase in *COL7A1* expression. This in turn is likely to stimulate an increase synthesis of mutant, but partially functional, collagen VII. However, the study does not define categorically the source of the new collagen VII and whether it originates from the donor fibroblasts or indirectly from recipient keratinocytes/fibroblasts but this has been addressed in previous studies (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003; Fritsch *et al.*, 2008; Kern *et al.*, 2009b; Conget *et al.*, 2010; Yan and Murrell, 2010). However, this study does outline a trend in the upregulation of

COL7A1 gene and protein expression which provides further insight into the pathways involved in the upregulation of *COL7A1* expression including immune responses that might act as the trigger for upregulation of *COL7A1*. It was suggested that perhaps the same mechanism apply to cases of bone marrow transplantation where the immune response to the transplantation rather than the bone marrow derived cells lead to upregulation of *COL7A1* expression (Uitto, 2011a). If proven then targeted therapy by the administration of HB-EGF can be trialled although this carries risks of tumourigenicity that needs to be taken into consideration particularly as RDEB patients have a significantly increased risk of developing squamous cell carcinoma (Kivisaari *et al.*, 2010). It also remains to be seen if EGF has similar signalling effects as HB-EGF with less effects on tumourigenicity.

Chapter 9

General Discussion

Great progress has been made over the last 2 decades in the clinical, immunohistochemical and molecular diagnostics of EB, including the discovery of the *COL7A1* gene on chromosome 3p21 as the gene underlying DEB (Ryynanen *et al.*, 1991), cloning of *COL7A1* (Christiano *et al.*, 1994b; Christiano *et al.*, 1994c; Parente *et al.*, 1991) and the subsequent identification of the first recessive *COL7A1* mutation; p.Met2798Lys and dominant mutation; p.Gly2040Ser in 1993 and 1994 respectively (Christiano *et al.*, 1994d; Christiano *et al.*, 1993). The subsequent plethora of published literature analysing the ultrastructural, molecular and clinical aspects of DEB has resulted in general paradigms for genotype-phenotype correlation and evolving classifications for EB that have seen the substitution of confusing historical eponyms with more clinically relevant ones. Phenotype-genotype correlation has been instrumental in making the various clinical distinctions, however, significant work stills need to be done, particularly in the field of DEB, to establish accurate and comprehensive paradigms that will address many yet unanswered questions. There is a need for deep phenotyping in association with comprehensive genotyping to answer questions such as: why is there considerable inter- and intra- familial heterogeneity and why do some mutations result in both dominant and recessive disease? What modifies disease expression to result in the various unique clinical subtypes such as EB pruriginosa? How can immunohistochemical and ultrastructural studies- the results of which are often non-specific- aid the process of genotype-phenotype correlation? And finally how can we optimize personalised and targeted therapies for DEB?

Studies outlined in this thesis have attempted to address some of these issues in a bid to refine genotype-phenotype correlation. Not only will this increase our understanding of DEB and the factors modulating phenotype expression but it will also have an impact on diagnosis, counselling, translational research and therapy, away from palliative medicine and towards personalised, specific and more definitive treatment.

In Chapter 3, the molecular, immunohistochemical and ultrastructural aspects of BDN, previously known as 'transient' BDN, were explored. Not only does BDN represent a distinct clinical entity with a very favourable outcome, but it also displays unique and striking features on immunofluorescence and electron microscopy that were worth addressing. The aim was to establish whether immunohistochemical and ultrastructural findings in these cases could provide an adjunct for the genotype-phenotype correlation. If so, then the relatively rapid immunofluorescence and electron microscopic studies could provide initial insight into subsequent disease severity aiding initial diagnosis and counselling. Moreover, the concept of intracytoplasmic collagen VII was previously generally synonymous with BDN. However, this Chapter sought to explore whether this finding was unique to this particular subtype of DEB.

The conclusion of Chapter 3, outlining one of the largest case series of BDN, highlights that intracytoplasmic retention of collagen VII is not exclusive to cases of BDN and can be associated with DDEB, RDEB, JEB, EBS as well as normal skin. This has also been supported by a recent study by Berk *et al*. (Berk *et al.*, 2013). Thus, on an immunohistochemical level, the finding of intra-epidermal retention of collagen VII could have diverse

diagnostic, phenotypic and prognostic implications. Similarly, variable ultrastructural patterns of dilated RER, including stellate bodies, have been elucidated on TEM not only in BDN but also in other forms of EB, as well as non-EB cases and even normal skin. Therefore, the definition of the ultrastructural stellate body, which has been used synonymously with BDN, should be referred to as a variant of dilated RER. In addition, correlation of TEM with IF studies should still be performed in all cases, as definitive diagnosis will always require prospective clinical assessments and mutational analysis.

Moreover, at the start of this thesis, GS mutations have been shown to underlie nearly half of dominant and recessive forms of DEB. In some cases there was unexplained overlap between the 2 inheritance patterns. In addition, a small number of non-GS mutations had been identified, mainly in RDEB but also in some DDEB cases. However, the genotype-phenotype correlation was far from characterised and the impact of these mutations on collagen VII expression was not quite clear. The key aims of Chapters 4 and 5 were to expand the collective *COL7A1* mutation database and to study whether the type or location of GS and non-GS mutations could predict phenotype expression and subsequently explain the genotype-phenotype heterogeneity encountered in DEB. Fifty seven novel mutations (40 GS and 17 non-GS mutations) were identified and analysed along with >200 published GS and non-GS mutations. Polymorphisms were ruled out by excluding mutations in 100 controls and performing *in silico* analysis where necessary. The novel mutations identified in this thesis have already been published and added to the various *COL7A1* specific databases including www.deb-central.org and www.col7.info.

Analysis of the findings in Chapters 4 and 5, in conjunction with the published literature was in keeping with the general paradigms established for DEB attributing dominant-negative mutations, particularly GS mutations to dominant forms of DEB cases and loss-of-function *COL7A1* mutations to its recessive counterpart. Analysis of my data confirmed frequent inter- and intra-familial heterogeneity that was further complicated by the finding of some GS mutations with both dominant and recessive modes of inheritance to which 4 novel mutations were added (12 in total).

However, no relation could be found between the type or location of GS and non-GS mutations and the clinical phenotype. Similarly, and largely owing to small numbers, no conclusion could be drawn about GS mutations resulting in both dominant and recessive disease, although a mixed pattern of inheritance is likely to exist. Of particular interest were those GS mutations resulting in a myriad of clinical phenotypes of variable severity including the more extensive DDEB and EB-pr as well as, nail dystrophy or no disease at all. One explanation could be related to the possibility of undetected mutations using the Sanger sequencing technique including larger deletion and insertions (Kern *et al.*, 2009a) which can lead to wrongly attributing a recessive disease to dominant mutations or even to erroneous diagnoses. This technical limitation is likely to explain the less than expected mutation pick up rate, which has been estimated in the EB National Diagnostic Laboratory in London at around 90% (Prof McGrath personal communication). Indeed, a recent study has highlighted the increased mutation rate in EB when new technology such as whole exome sequencing is used in a diagnostic setting. Therefore, the diagnosis of RDEB -and EB in general- should not be excluded based on non-

confirmatory molecular analysis particularly where there is strong clinical suspicion.

Phenotype heterogeneity could also be explained by genetic, epigenetic or even environmental factors. Unpredicted consequences of nucleotide substitutions on splicing as a result of proximity to exonic splicing enhancers (ESE) for example, can affect protein expression, as can biochemical effects on collagen VII by altering helix formation, protein folding, thermal stability, intracellular transport, secretion, and assembly into anti-parallel dimers or anchoring fibrils (Shimizu *et al.*, 1996; Christiano *et al.*, 1996a; Hammami-Hauasli *et al.*, 1998b; Lee *et al.*, 2000; Sato-Matsumura *et al.*, 2002; Kern *et al.*, 2006; Chiaverini *et al.*, 2010; Chen *et al.*, 2001; Chen *et al.*, 2002a; Brittingham *et al.*, 2005; Fritsch *et al.*, 2009; Hyde *et al.*, 2006). Other influencing factors include the effects of endoplasmic reticulum stress and mutant proteins on apoptosis and gene expression, as well as gene promoter polymorphisms and transcriptional regulators such as *MMP-1* and the high mobility group box 1 (HMGB1) respectively, which were found to correlate with disease severity in RDEB (Petrof *et al.*, 2013a).

Taking this into consideration, it becomes clear that although the type and location of *COL7A1* mutations might offer general paradigms for genotype phenotype correlation, the missing piece of the puzzle is likely to lie beyond the *COL7A1* gene and within genetic and epigenetic modulators that are likely to become evident with the introduction of whole genome and exome sequencing as well as data mining studies. This is supported by emerging data from the ENCODE (encyclopaedia of DNA elements) project suggesting that >80% of the human genome including non-coding DNA contains functional

elements that could influence gene expression compared to the previously thought 1.2% exonic DNA (ENCODE Project Consortium *et al.*, 2012). An interplay between multiple genetic, epigenetic and environmental modifiers is likely to be involved and further functional studies applying proteomic and transcriptomic principles are needed to verify the effects of the various modulators on gene expression as well as protein folding, secretion and degradation.

Therefore, refining genotype-phenotype correlation based on *COL7A1* analysis alone and in the absence of detailed information on genetic, epigenetic and environmental modifiers will prove to be difficult and suboptimal. However, attempts to understand disease expression as outlined in this thesis, might offer minor refinement and improvement to the general genotype-phenotype correlation that might also help to identify distinct subtypes of DEB amenable for targeted analysis and scrutiny. One example is the *MMP-1* promoter polymorphism, whose role in the itchy EB-pr phenotype was studied in Chapter 6. As outlined in this study, examination of the GS mutations associated with EB-pr - including 6 novel mutations- revealed that the nature of the pathogenic *COL7A1* mutations in EB-pr did not seem to differ from those found in patients with dominant DEB subtypes without pruritus. In addition, although the findings in this Chapter support the published evidence that the -1607 1G/2G polymorphic variant in the *MMP1* promoter may be associated with a more severe RDEB phenotype, similar effects on its dominantly inherited counterpart were not identified. There were no significant differences in the frequency of the -1607 1G/2G *MMP-1* promoter polymorphism between patients with dominantly inherited EB-pr and those with other forms of DDEB

or controls and therefore it is unlikely to play a role in the modulation of the pruritic EB-pr phenotype. Since the publication of this study, the above results were supported by a subsequent publication by Kern *et al.* in which no correlation was found between the *MMP-1* 2G promoter polymorphism and disease severity in DDEB (Kern *et al.*, 2009a). In addition, no relation was found to RDEB severity or the development of aggressive SCC. Therefore what accounts for the unique pruritic phenotype of EB-pr remains unclear as evident by more recent longitudinal studies and case series (Fortuna *et al.*, 2013; Brick *et al.*, 2012; Tang *et al.*, 2013; Takiyoshi *et al.*, 2012; Covaciu *et al.*, 2011), although in one recent study co-infestation with scabies was highlighted as a potential environmental modifier triggering the pruritic EB-pr phenotype (Kim *et al.*, 2013). The cytokine IL-31 that plays a role in atopic eczema was not found to be implicated in EB-pr (Nagy *et al.*, 2010). Unfortunately, this lack of disease explanation is an impediment to the development of direct and specific anti-pruritic therapy for EB-pr. Several agents have been explored in the past including ciclosporin and thalidomide with disappointing results and the effect of others recently trialled in eczema is yet to be established in EB-pr including the immunomodulators apremilast; a phosphodiesterase 4 inhibitor and dupilumab; a monoclonal antibody that inhibits both IL-4 and IL-13 signalling (Harskamp and Armstrong, 2013).

However, although the 'inside-out' approach to genotype-phenotype correlation scrutinizing the implications of molecular, immunohistochemical and ultrastructural findings on clinical expression as outlined in Chapters 3-6 has been integral to genotype-phenotype correlation in EB, an exciting concept with tremendous pathophysiological and therapeutic implications is the

‘outside-in’ dimension represented by somatic mosaicism, where the observation of normal patches of skin reflects the presence of natural gene correction within a subpopulation of cells. Prior to this thesis, reports of RM in DEB did not exist, however the RM phenomenon was described in other forms of EB including nHJEB (Darling *et al.*, 1999; Pasmooij *et al.*, 2005; Pasmooij *et al.*, 2007; Jonkman and Pasmooij, 2009; Jonkman *et al.*, 1997) and EBS (Schuilenga-Hut *et al.*, 2002; Smith *et al.*, 2004).

In Chapter 7, the first case of revertant mosaicism in DEB was studied. This revealed intragenic cross-over in the *COL7A1* gene as the mechanism underlying RM in this case. The correction, which occurred in keratinocytes and not fibroblasts, resulted in subsequent generation of wild type alleles and restoration of collagen VII expression at the basement membrane. Anchoring fibrils were also detectable in reverted skin. Therefore, it is likely that the RM phenomenon, which was historically exclusive to nHJEB cases, is more common than initially thought as evidenced by the publication of 3 subsequent cases of RDEB and Kindler syndrome with proven RM (Pasmooij *et al.*, 2010; van den Akker *et al.*, 2012; Lai-Cheong *et al.*, 2012). Pasmooij *et al.* reported a case of RM due to a correcting somatic *COL7A1* nucleotide deletion; c.6528delT (Pasmooij *et al.*, 2010). This also occurred in keratinocytes only and led to the occurrence of an expanding revertant patch but it was not clear whether this occurred during embryogenesis or later in life. More recently, van den Akker *et al.* described another case with a nucleotide substitution c.6510G>T that reverted the nonsense mutation p.Gln2170Stop to p.Gln2170Tyr resulting in amelioration of collagen expression (van den Akker *et al.*, 2012). Therefore, the publication of 3 cases of RDEB with RM within a

short space of time is probably a reflection of a not-so-rare phenomenon that is under reported.

However, further work needs to be done to understand whether the RM phenomenon is a random event or is triggered by genetic or environmental factors. In support of the latter is the observation that RM generally occurs in high turnover tissue and is not noted at birth. Furthermore, it is still not clear whether RM arises in the skin through unknown events or in epithelial progenitor cells in the bone marrow that are subsequently recruited to injured skin via signalling mediators such as HMGB1. The latter might explain the multiple and simultaneous appearance of revertant patches. In addition, understanding of the timing at which reversion occurs and the predilection for keratinocytes and not fibroblasts will have clinical implications particularly when it comes to harnessing this phenomenon for therapeutic purposes.

Nevertheless, the discovery of RM in RDEB is exciting as it provides a much-needed model for the study of natural gene correction mechanisms in DEB that could potentially be recapitulated *in vivo*. Also it offers an endless resource of autologous patient specific cell therapy, including localised cell therapy such as punch grafting (Gostynski *et al.,* 2014), BM transplantation and generation of iPSCs (Aasen *et al.*, 2008; Tolar *et al.*, 2009; Tolar *et al.*, 2011; Itoh *et al.*, 2011; Jonkman and Pasmooij, 2012). The reprogramming of revertant cells into iPSCs capable of differentiating into autologous keratinocytes would preclude the use of retroviral vectors for gene correction and would circumvent issues related to rejection (Itoh *et al.*, 2011; Tolar *et al.*, 2011; Tolar *et al.*, 2014; Woodley *et al.*, 2013a). iPSCs can also be differentiated into hematopoietic or mesenchymal stem cells obviating the need

for risky BM transplantation.

The discovery of RM in DEB is just an example of the advances made in the last couple of decades in terms of understanding the pathophysiology of DEB and its underlying molecular, immunohistochemical and ultrastructural associations. Yet for a long time this has not been paralleled by advances in therapy, which until recently have been largely preventative or palliative. At the time this thesis began, only a limited experimental medications were tried. However, in the last few years, significant developments have been made in the field of translational medicine involving protein, cell and gene therapy. Earlier proof of principal studies using pre-clinical models, have led to the implementation of several clinical trials with promising results. Ironically, advances in therapy have also provided further insight into DEB pathophysiology. For example, cell therapy and as demonstrated in Chapter 8, does not only result in clinical amelioration of skin fragility but also provides a model from which we could elucidate which mediators and genetic modifiers influence collagen VII expression when the inducing cells are no longer present.

At the start of this thesis, fibroblasts were used in DEB as an off label therapy. In a study by Wong *et al.* in 2008 a single intradermal injection of 5 $x10^6$ allogeneic human fibroblasts in non-blistered RDEB skin was found to increase collagen VII expression and anchoring fibril formation, for up to three months (Wong *et al.*, 2008). The key questions arising from that study were regarding the mechanism behind this clinical and ultrastructural amelioration and the lasting effects of allogeneic fibroblast injections.

The follow-on study in Chapter 8 was carried out in 1 patient from the previous study by Wong *et al.* This patient, known to be compound heterozygous for the *COL7A1* nonsense mutation c.2044C<T (p.Arg682Stop) and the splice site mutation IVS87+4A>G, was chosen because she showed higher levels of collagen VII expression at the DEJ at baseline compared to the other subjects and was subsequently shown to develop a higher collagen VII expression following allogeneic fibroblast injection, which was sustained for 3 months. Following the intradermal injection of 5 x 10^6 allogeneic fibroblasts per cm^2, the effects were analysed for up to 360 days and results demonstrated that a single injection is capable of increasing collagen VII labelling at the DEJ by almost 1.5 fold and that this was maintained for >9 months. *COL7A1* gene expression was upregulated for >3 months and was mirrored by an increase in the expression of HB-EGF. Subsequent *in vitro* incubation of keratinocytes and fibroblasts with HB-EGF revealed an associated increase in *COL7A1* expression, potentially via increased AP-1 transcription factor. It is likely that allogeneic fibroblasts (and saline to a much lesser extent) and through induction of subclinical inflammation leads to the upregulation of HB-EGF. This in turn is likely to stimulate an increased synthesis of mutant, but partially functional, collagen VII. However, the study does not define categorically the source of the new collagen VII and whether it originates from the donor fibroblasts or indirectly from recipient keratinocytes/fibroblasts but this has been addressed in previous studies (Ortiz-Urda *et al.*, 2003; Woodley *et al.*, 2003; Fritsch *et al.*, 2008; Kern *et al.*, 2009b; Conget *et al.*, 2010; Yan and Murrell, 2010). However, what this study does outline is a trend in the upregulation of *COL7A1* gene and protein expression following allogeneic

fibroblast injection, which provides further insight into the pathways involved in *COL7A1* expression including the potential role of immune responses. The latter, as suggested in a commentary by Prof. Uitto might have implications on bone marrow transplantation in RDEB, where the immune response to the transplantation procedure itself rather than the bone marrow derived cells might be what leads to upregulation of *COL7A1* expression (Uitto, 2011a). If proven, then optimized and targeted therapy utilizing key-molecules that modulate *COL7A1* expression such as HB-EGF can be trialled. However the risks of tumourigenicity would need to be taken into consideration particularly as RDEB patients have a significantly increased risk of developing squamous cell carcinoma (Kivisaari *et al.*, 2010). It also remains to be seen if EGF has similar signalling effects as HB-EGF with less effects on tumourigenicity.

Since the completion of the above study, several clinical trials have taken place and several are ongoing involving allogeneic fibroblast therapy in humans (Petrof *et al.*, 2013b; Venugopal *et al.*, 2013). In a recent study by Petrof *et al.* a single intradermal injection of allogeneic fibroblasts in subjects with RDEB was shown to increase the initial rate of erosion healing within the first 28 days but not thereafter (Petrof *et al.*, 2013b). In another study by Venugopal *et al.* the injection of either allogeneic fibroblasts or suspension solution alone in patients with RDEB-sev gen was shown to improve wound healing in chronic wounds independently of collagen VII expression (Venugopal *et al.*, 2013). However, studies exploring the effects of cell therapy on chronic wounds should be put in context with the normal healing process occurring in RDEB and a longitudinal study looking into this is yet to be conducted. Therefore, an ideal prospective trial exploring cell therapy should

involve a 3-arm study comparing the effects of injectable cells vs. vehicle compared to untreated wounds. Furthermore, studies are needed to address the potential benefit of re-treatment as well as the optimal mode of cell delivery.

More recently, stem cell therapy has emerged as a unique method of treatment owing to the plasticity and regenerative potential of stem cells with a capacity to differentiate into keratinocytes as well as fibroblasts. Moreover, it has been shown that in addition to the bone marrow, effective stem cells can be derived from various sources including the readily accessible adipose tissue and cord blood or through induction of iPSCs. The latter refers to the reprogramming of somatic cells by the use of a multitude of transcription factors into embryonic stem cells capable of differentiating into epithelial cells and fibroblasts (Aasen *et al.*, 2008; Aasen and Izpisua Belmonte, 2010; Takahashi and Yamanaka, 2006; Uitto, 2011b; Tolar *et al.*, 2011; Bilousova *et al.*, 2011).

Intradermal infusion of allogeneic BM derived mesenchymal stromal cells (MSCs) in RDEB patients resulted in increased collagen VII expression at the DEJ and improved healing of chronic ulcers (Conget *et al.*, 2010). Similarly, preclinical studies in *COL7A1* null animal models demonstrated that the systemic infusion of bone marrow derived stem cells resulted in amelioration of collagen VII expression and subsequent reduction of skin fragility. Survival was also extended in the treated mouse embryos (Tolar *et al.*, 2009; Chino *et al.*, 2008).

These studies paved the way for clinical trials using infusions of allogeneic bone marrow derived stem cells in children with RDEB following chemoablative pre-conditioning. This led to partial correction of collagen VII

expression with amelioration of skin fragility. However, this procedure is fraught with risks relating to immunosuppression. Therefore, measures currently being assessed to reduce morbidity and mortality include use of reduced intensity chemoablation and co-infusion of mesenchymal stem cells (Uitto *et al.*, 2012; Petrova *et al.*, 2010; Conget *et al.*, 2010). Another consideration is the infusion of homologous transgenic stem cells or patient specific-RDEB iPSCs as a way to achieve safer patient specific therapy (Tolar *et al.*, 2011; Murauer *et al.*, 2011; Uitto, 2011b). RDEB iPSCs derived from fibroblasts and keratinocytes have been used to generate both mesenchymal and haematopoietic stem cells that can produce collagen VII *in vivo* (Tolar *et al.*, 2011; Uitto, 2011b; Bilousova *et al.*, 2011). A significant draw back with the use of iPSCs however, relates to the increased risk of malignancy owing to the use of transcription factors that could potentially lead to oncogenic effects (Liang *et al.*, 2012).

More recently, *in vivo* studies have demonstrated that local and systemic injection of human umbilical cord blood-derived unrestricted somatic stem cells (USSCs) resulted in wound healing and skin regeneration in mouse models. USSCs, even if injected intradermally at a distant site, were capable of migrating to areas of wounding (Liao *et al.*, 2013).

Therefore stem cell therapy is an exciting therapeutic platform with potential to provide sustained and widespread correction of collagen VII expression if applied earlier in life. However, the next challenge lies in ascertaining whether the amelioration of collagen VII expression is related to increased wild type collagen VII production by transplanted cells or as a result of increased production of mutant yet partially functional collagen VII

(Prockop, 2009; Tolar *et al.*, 2010; Chen *et al.*, 2008; Nauta and Fibbe, 2007). Also it is still not clear which specific progenitor effector cells are capable of collagen VII production and the mechanism by which its expression is induced. So far, studies have established that BM cells homing to skin are derived from haematopoietic or mesenchymal stromal cell populations with an ability to differentiate into keratinocytes and also fibroblast-like cells (Badiavas *et al.*, 2003; Borue *et al.*, 2004; Fathke *et al.*, 2004; Abdul-Wahab *et al.*, 2012). However, the exact nature of this subpopulation of progenitor cells is yet to be determined. Recent work by Tamai *et al.* has established that a subpopulation of bone-marrow platelet-derived growth factor receptor α-positive (Lin−/PDGFRα+) MSCs contained epithelial progenitors. These cells were also found to be mobilised into wounded skin by HMGB1 (Tamai *et al.*, 2011), a nuclear protein capable of modulating gene expression (Harris and Raucci, 2006), as supported by the above study, where HMGB1 was shown to facilitate recruitment of BM subpopulations with epithelial regenerative potential into wounded skin leading to increased local expression of *COL7A1* (Tamai *et al*., 2011). This provides exciting insight into mechanisms of stem cells differentiation and migration with implications for cell and drug therapies.

Since the publication of the study outlined in Chapter 8, leaps have been made in the field of gene, drug, protein and cell therapy in EB that have seen the application of silencing small molecules and SMarT technologies (Chamcheu *et al.*, 2012), as well as engineered transcription activator-like effector nucleases (TALEN) and clustered regulatory interspaced short palindromic repeats (CRISPR) associated RNA-guided DNA endonucleases for precise genome editing *in vivo* to increase collagen VII expression

(Pendaries *et al.*, 2012; Murauer *et al.*, 2011; Murauer *et al.*, 2013; Turczynski *et al.*, 2012; Osborn *et al.*, 2013; Gruber *et al.*, 2013; Umov *et al.*, 2010; Carroll, 2011). Not only will these technologies inadvertently lead to an increase in the functional level of wild type collagen VII but they will also circumvent the use of retroviral vectors providing a safer and more patient specific therapeutic option.

With regards to protein therapy and since the completion of this thesis, intradermal injection of purified recombinant collagen VII was attempted in collagen VII null mice. However, this treatment was limited by the extensive mucosal and cutaneous involvement in DEB, as well as the limited diffusion of intradermally injected collagen VII (Woodley *et al.*, 2013b). In addition, this approach was associated with the formation of antibodies to the foreign collagen VII protein but in another study anti-CD40L monoclonal antibody has been utilised with success in blocking this reaction (Remington *et al.*, 2009). Moreover, in a recent study by Woodley *et al.* 12 out of 22 patients with RDEB were found to have low level of circulating anti- collagen VII autoantibodies. However, the antibodies did not bind to the DEJ and were thought to be non-pathogenic (Woodley *et al.* 2014).

In a recent study by Woodley *et al.*; recombinant collagen VII was injected intravenously in 2 animal models; athymic nude mice with full thickness skin wounds, as well as an RDEB skin transplantation mouse model. Recombinant collagen VII incorporated into the DEJ after 2 weeks and was sustained for 8 weeks post injection, leading to immunohistochemical and ultrastructural amelioration of collagen VII expression and improved skin fragility and healing (Woodley *et al.*, 2013b). Topical application of

recombinant collagen VII was also attempted in animal models with similar results (Wang *et al.*, 2013).

From a therapeutic perspective, microneedling is being explored in a randomized controlled trial as a way to promote wound healing in DEB (Australian New Zealand Clinical Trials Registry). Etanercept (Gubinelli *et al.*, 2010) and mycophenolate mofetil (Eldarouti *et al.*, 2013) were used in RDEB patients with some success, however, more specific drug therapies have recently emerged with promising results. Some, such as aminoglycosides took advantage of the proofreading properties leading to read-through correction of mRNA bearing premature termination codons (PTCs) (Rowe and Clancy, 2009). Others targeted potential disease modifiers such as aberrant splicing. The histone deacetylase inhibitor; sodium butyrate (NaBu), has been shown to modulate the expression of splicing factors with subsequent implications on functional protein expression (Garcia-Blanco *et al.*, 2006; Nissim-Rafinia *et al.*; Kerem *et al.*, 2005). Also, inhibition of MMP-1, a collagenase that degrades collagen VII could potentially lead to amelioration of protein expression in severe RDEB (Titeux *et al.*, 2008).

The progress in drug therapy has also been accompanied by advances in the modes of delivery of various treatments including topical applications, as well as local and intravenous injections. More recently, nanotechnology has emerged as a promising new application for drug delivery. This utilises nanoparticle systems to increase bioavailability but without unwanted side-effects (Chamcheu *et al.*, 2012). If published results are reproducible, then this technology has the potential of enhancing the delivery of any novel drug therapy with increased tolerability and efficacy.

Moreover, advances in stem cell biology including the identification of stem cell subpopulations contributing to skin recovery and mechanisms involved in tissue regeneration, as well as the recognition of modulating cytokines and transcription factors, will enable the use of specific and targeted molecules capable of recruiting effector progenitor cells into diseased skin. Furthermore, the use of iPSCs as a model to recapitulate EB disease mechanisms will be instrumental in identifying similar drug targeting pathways.

In conclusion, at the heart of EB gene discovery, translational research and personalized medicine lies the genotype-phenotype correlation. Not only has this triggered the analytical questioning behind this thesis and EB research in general but it is likely to continue to modify how we approach EB, whether it is by adapting classifications, recognizing the role of genetic, epigenetic and environmental modulators or through optimization of patient specific therapies. Although studies carried out in this thesis offered minor refinement to the general genotype-phenotype correlation, they identified key repair mechanisms for natural gene therapy and showed that a single growth factor can increase *COL7A1* expression and protein synthesis. Several directions for the future have been mentioned in the general discussions sections, however, it is clear that significant improvement to the genotype-phenotype correlation will require greater insight into gene-gene and gene-environment modulation, likely to be provided in the near future by emerging data from the ENCODE project. Molecular, proteomic and transcriptomic studies will be needed to subsequently characterise and validate newly-found pathogenic modulators and their effects on disease expression, while phenotype correlation will continue

to form the basis of our clinical questioning and on which we base future therapies.

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